UNIVERSIDAD AUTÓNOMA DE MADRID

FACULTAD DE CIENCIAS

DEPARTAMENTO DE QUÍMICA-FÍSICA APLICADA

Sección Departamental de Ciencias de la Alimentación

Instituto de Investigación en Ciencias de la Alimentación (CIAL)

CIAL

SÍNTESIS DE FOSFOLÍPIDOS PORTADORES PARA LA OBTENCIÓN DE COMPUESTOS BIOACTIVOS

Víctor Casado Bañares

Tesis doctoral

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Memoria presentada por:

Víctor Casado Bañares

Para optar al grado de:

DOCTOR EN CIENCIA Y TECNOLOGÍA DE LOS ALIMENTOS

Trabajo realizado bajo la dirección de:

Dr. Carlos F. Torres Olivares

(Universidad Autónoma de Madrid)

Don Carlos Torres Olivares Dr. en Química y Profesor Titular de Ciencia y Tecnología de Alimentos de la Universidad Autónoma de Madrid.

INFORMA:

Que el presente trabajo titulado “Síntesis de fosfolípidos portadores para la obtención de compuestos bioactivos” y que constituye la memoria que presenta D. Victor Casado Bañares para optar al grado de Doctor en Ciencia y Tecnología de los Alimentos, ha sido realizado en el Instituto de Investigación en Ciencias de la Alimentación UAM-CSIC bajo su dirección.

Autorizan expresamente la presentación de la Tesis Doctoral como un compendio de publicaciones.

Y para que así conste firma el presente informe en Madrid a 10 de Marzo de 2014.

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ÍNDICE

1 ÍNDICE

1 RESUMEN

En esta memoria, se muestran los resultados más relevantes obtenidos de la producción de fosfolípidos estructurados, estos nuevos compuestos tienen propiedades saludables, un alto valor añadido y pueden ser utilizados en la elaboración de alimentos funcionales y nutracéuticos. Dos fosfolípidos estructurados con tirosol e hidroxitirosol han sido sintetizados y purificados. Las dos principales metodologías utilizadas han sido elegidas por su carácter respetable con el medio ambiente, estas son; la síntesis enzimática y la extracción con disolventes GRAS, ambas idóneas para la obtención de productos de grado alimentario.

En primer lugar, se ha analizado el desarrollo tecnológico existente en el actual sector industrial alimentario, haciendo hincapié en el uso de fosfolipasas en diferentes campos de la elaboración de alimentos. De la misma manera se ha estudiado, la tecnología de lípidos portadores en el ámbito científico, para valorar el grado de innovación requerido. Seguidamente, se ha desarrollado la producción de derivados fosfolipídicos de tirosol e hidroxitirosol basándose en una estrategia que contempla sostenibilidad, aplicabilidad y rentabilidad del proceso en el sector alimentario. Por tanto, parámetros como temperatura, medios de reacción, relación entre sustratos, concentración de sustratos, concentración de enzima, relación sustratos-enzima o escalado han sido optimizados, con el fin de conseguir una alta productividad. El diseño y la optimización de las reacciones desarrolladas, han sido influenciadas por la posterior extracción del producto sintetizado, influyendo esta fase en la toma de decisiones para conseguir una purificación fácilmente aplicable al sector industrial. Es importante destacar, que este trabajo ha sido desarrollado valorando su potencial aplicación en procesos industriales y todos los resultados han sido obtenidos a escala de planta piloto.

2 LISTA DE ABREVIATURAS

AGs: Ácidos grasos

DGs: Diglicéridos

DHA: Ácido graso docosahexaenoico

EPA: Ácido graso eicosapentaenoico

HT: Hidroxitirosol

LPs: Lisofosfolípidos

LPA: Ácido lisofosfatídico

LPC: Lisofosfatidilcolina

LN: Lípidos neutros

PA: Ácido fosfatídico

PC: Fosfatidilcolina

PE: Fosfatidiletanolamina

PG: Fosfatidilglicerol

PHT: Fosfatidilhidroxitirosol

PT: Fosfatidiltirosol

PI: Fosfatidilinositol

PLs: Fosfolípidos

PLA: Fosfolipasa A

PLC: Fosfolipasa C

PLD: Fosfolipasa D

PS: Fosfatidilserina

PT: Fosfatidiltirosol

SEEDS: Sistemas autoemulsionables

SM: Esfingomielina

TGs: Triglicéridos

3 ESTRUCTURA DE LA MEMORIA

El procedimiento relativo al tribunal, defensa y evaluación de la tesis doctoral en la Universidad Autónoma de Madrid, aprobado en Consejo de Gobierno el 3 de febrero de 2012, permite presentar la tesis doctoral como un compendio de publicaciones. Para ello se requiere un mínimo de 3 artículos publicados o admitidos para su publicación en revistas científicas de reconocido prestigio o en libros editados de importancia justificada. La memoria del trabajo de Tesis Doctoral debe incluir una introducción general que presente los trabajos compendiados, justifique la temática y explique la aportación original del autor, así como un resumen global de los resultados obtenidos, de la discusión de éstos y de las conclusiones finales. Se ha de incluir una copia completa de los mencionados trabajos.

La memoria de la presente investigación de Tesis Doctoral responde a los requisitos anteriormente descritos y está estructurada en seis secciones. Dentro del apartado de resultados y discusión, se han incorporado cuatro artículos científicos realizados en torno a la obtención de fosfolípidos portadores bioactivos, que han sido publicados en revistas de difusión científica internacional.

1. Presentación y Objetivos: planteamiento de las hipótesis del plan de trabajo, así como los objetivos generales y específicos de esta memoria.

2. Introducción: presentación de los fundamentos y antecedentes correspondientes a los trabajos realizados.

3. Plan de trabajo: descripción de la metodología y los procedimientos aplicados, y las tareas realizadas para alcanzar los objetivos planteados.

4. Resultados y Discusión: Publicaciones presentadas en inglés y con el esquema convencional de las publicaciones científicas.

- Carlos Torres, Diana Martín, Guzmán Torrelo, Víctor Casado, Oscar Fernández, Daniel Tenllado, Luis Vázquez, Inés Morán-Valero, Guillermo Reglero. (2011) "Lipids as delivery systems to improve the biological activity of bioactive ingredients." Current Nutrition and Food Science 7(3): 160-169

- Víctor Casado, Diana Martín, Carlos Torres, Guillermo Reglero. (2012). "Phospholipases in Food Industry: A Review". Lipases and Phospholipases. Methods in Molecular Biology series, G. Sandoval, Humana Press. 861: 495-523.

- Víctor Casado, Guillermo Reglero, Carlos Torres. (2013). "Production and Scale-up of phosphatidyl-tyrosol catalyzed by a food grade phospholipase D." Food and Bioproducts Processing 91(4): 599-608.

- Víctor Casado, Guillermo Reglero, Carlos Torres. (2014). "Novel and efficient solid to solid transphosphatidylation of two phenylalkanols in a biphasic GRAS medium." Journal of Molecular Catalysis B: Enzymatic 99(0): 14-19.

5. Discusión general: se presenta una discusión general de los trabajos presentados en la memoria, relacionando los resultados obtenidos en las distintas sub-secciones.

6. Conclusiones: presentación de las conclusiones generales y más relevantes obtenidas en los trabajos expuestos y discutidos en las secciones anteriores.

4 PRESENTACIÓN Y OBJETIVOS

Los hábitos alimentarios pueden describirse como patrones rutinarios de consumo en la dieta. En las últimas décadas, se han creado nuevas tendencias en dichos hábitos y sobre todo existe una nueva concepción del significado de los alimentos. El actual interés por seguir una dieta sana para alcanzar estados óptimos de salud, ha dado lugar a estas nuevas tendencias en consumo de alimentos, que van dirigidas a aportar beneficios para la salud, además de ser seguros y nutritivos. El motor de estos cambios es el nuevo estilo de vida propio de las sociedades desarrolladas. Sin embargo, la falta de tiempo para cocinar, el ritmo de vida actual y la enorme oferta de alimentos hacen complicada la toma de decisiones adecuadas y conduce a que muchas personas no sigan una alimentación equilibrada. Por lo tanto, aunque es cierto que se ha producido un aumento del interés por los problemas dietéticos relacionados con la salud y con el consumo de los llamados alimentos sanos, es necesaria, una mayor claridad tanto en las propiedades de los alimentos, como en sus procesos de elaboración, de modo que se ayude al consumidor a elegir determinados alimentos y a excluir otros.

En esta tesis se pretende contribuir al avance de la tecnología alimentaria, específicamente en la obtención de lípidos portadores, mediante la utilización de biocatálisis con fosfolípidos. Los lípidos portadores pueden mejorar la incorporación a matrices alimentarias o mejorar la biodisponibilidad y actividad biológica de diversos compuestos bioactivos. La estrategia de los lípidos portadores ha sido desarrollada principalmente por el campo farmacéutico, en la liberación de principios activos. Pero la aplicación en el campo de la alimentación está en fase de desarrollo, proporcionando la optimización de absorción de ingredientes bioactivos por el organismo.

El objetivo general de esta tesis es obtener fosfolípidos estructurados que incorporen compuestos fenólicos bioactivos, mediante el empleo de tecnologías inocuas y medioambientalmente limpias, para su posterior aplicación en el campo de la alimentación.

La producción de fosfolípidos estructurados bioactivos, mediante el uso de fosfolipasas y particularmente de fosfolipasa D ha sido descrita en el campo científico en diversas

ocasiones. Sin embargo, el uso de disolventes tóxicos, el cual conlleva riegos para el ser humano está muy extendido en este campo. Por otro lado, las concentraciones de los sustratos en dichas reacciones son bajas, por lo tanto, las cantidades de productos obtenidos son a su vez relativamente bajas en la mayoría de los casos. De la misma manera, en los trabajos a escala laboratorio, la rentabilidad del proceso no suele ser valorada, obviando por ejemplo, la relación sustratos/enzima utilizada.

Una serie de objetivos específicos también han sido propuestos:

- **Estudiar las diferentes fosfolipasas disponibles, tanto comercialmente como en el ámbito científico, para conseguir diseñar modificaciones de fosfolípidos viables en la industria alimentaria**.
- **Fortalecer las metodologías de extracción y análisis de muestras lipídicas que contienen fosfolípidos, orientadas al seguimiento de las reacciones de transformación de fosfolípidos y la obtención de fosfolípidos estructurados.**
- **Desarrollar un proceso biocatalítico, en el que ninguno de los componentes de la reacción sea potencialmente peligroso para el ser humano, utilizando enzimas y disolventes GRAS.**
- **Conseguir alta productividad en la síntesis del fosfolípido estructurado para asegurar su rentabilidad, con el fin de estudiar la viabilidad de la aplicación en el sector industrial alimentario.**
- **Aislar el fosfolípido estructurado de forma sencilla y sin utilizar disolventes tóxicos, para conseguir obtener un producto en polvo, sin humedad y lo más puro posible.**
- **Conseguir ajustar el proceso desarrollado en planta piloto, para valorar los problemas que surgen en el escalado.**

INTRODUCCIÓN

5 INTRODUCCIÓN

5.1 FOSFOLÍPIDOS

5.1.1 Bioquímica del fosfolípido

Los fosfolípidos (PLs) son un tipo de lípido alimentario ingerido a diario en nuestra dieta, pero poco conocido por la mayoría de los consumidores. Históricamente todos los lípidos han tenido connotaciones negativas para la salud, aunque estos son imprescindibles para la vida. Actualmente, sabemos que los lípidos no sólo son necesarios, sino que, tomadas en su justa medida, y dependiendo del tipo que sean, son consideradas saludables. Este es el caso de los PLs, que son los principales constituyentes de las membranas celulares y juegan un papel crucial en la bioquímica y fisiología de la célula. Los principales roles de los PLs en el organismo son:

-Ofrecer garantías estructurales para la integridad de las membranas y ayudar a llevar a cabo sus funciones. Su estructura molecular, con carácter anfipático, permite la constitución de la bicapa lipídica esencial para la membrana celular [1, 2].

-Regular el metabolismo y fisiología de la célula, ya que constituyen una importante fuente de precursores para mediadores y mensajeros altamente activos, tales como eicosanoides, diacilglicerol, inositol fosfatos o factores de activación plaquetaria [3].

-Regular la señalización celular, debido a que, la cabeza polar y diversos ácidos grasos (AG), pueden ser liberados para generar segundos mensajeros que son utilizados en transducción de señales en la célula. Los PLs participan en la transducción de señales biológicas a través de la membrana y median en una gran cantidad de eventos dentro y fuera de la célula[4-6].

-Regular procesos biológicos básicos como absorción intestinal de lípidos y síntesis de prostaglandina y lipoproteínas [7].

-Proporcionar energía y ácidos grasos poliinsaturados como araquidónico, eicosapentaenoico [8] y docosahexaenoico (DHA), jugando un importante papel en el crecimiento, desarrollo cerebral y células de la retina [4].

5.1.2 Clasificación y estructuras de los fosfolípidos

Existe una gran variedad de PLs en la naturaleza, pero generalmente todos ellos contienen una cadena de glicerol o de esfingosina [9].

Usualmente se conoce como PLs a los componentes del subgrupo de glicerofosfolípidos (GPLs). Estos GPLs se caracterizan por contener un esqueleto de glicerol, AGs en posiciones 1 y 2, un grupo fosfato y unido a este, un alcohol. Sin embargo, los PLs se clasifican en cuatro clases principales: los GPLs ya mencionados, esfingolípidos, eterfosfolípidos y fosfonolípidos, en función de su cadena principal y sus tipos de enlaces [10, 11].

Glicerofosfolípidos

Los GPLs son lípidos abundantes en la naturaleza, contienen una parte apolar formada por el grupo diacilo y una porción polar por el grupo fosfato, al que se une un grupo alcohol, como se presenta en la Figura 1. Los GPLs se clasifican en función de la naturaleza de los AGs y el alcohol unido al grupo fosfato, así como de las propiedades y las funciones biológicas de los GPLs. Cuando estas estructuras contienen una colina, serina, etanolamina, glicerol o inositol son nombrados como fosfatidilcolina (PC), fosfatidilserina (PS), fosfatidiletanolamina (PE), fosfatidilglicerol (PG), fosfatidilinositol (PI).

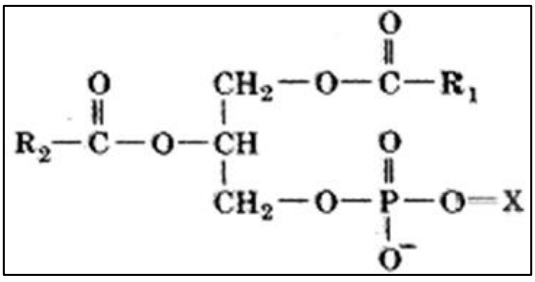


Figura 1. Estructura general del glicerofosfolípido

Existen tres tipos de moléculas con variantes en su estructura con gran repercusión en el metabolismo humano (Figura 2):

1) Aquellos que tan sólo tienen un ácido graso en posición 1 o en posición 2 del glicerol denominados lisofosfolípidos (LPs) (monoacilglicerofosfolipido). 2) El ácido fosfatídico (PA), que es el fosfolípido sin ningún alcohol unido al grupo fosfato. 3) El compuesto que contiene dos grupos diacilglicerofosfatidil unidos en la posición 1 y 3 de la misma molécula de glicerol, llamado cardiolipina.

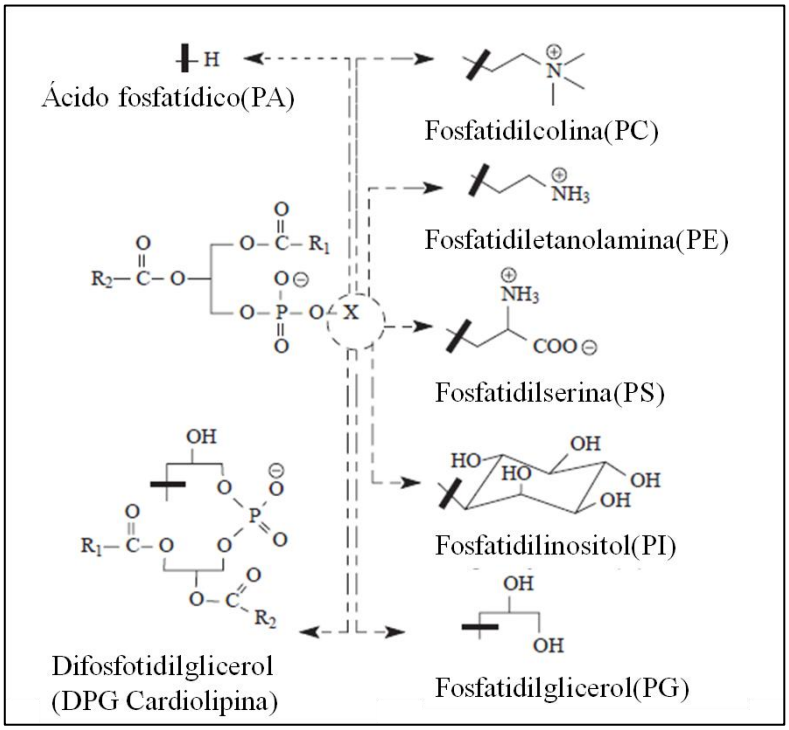


Figura 2. Representación esquemática de las estructuras químicas de glicerofosfolípidos [10]

Esfingolípidos

Los esfingolípidos son un amplio grupo de lípidos complejos cuya estructura es una cadena larga de aminoalcohol, comúnmente trans-1,3-dihidroxi-2-amino-4-octadeceno, (esfingosina en mamíferos) [12, 13].

Pero la estructura de las moléculas de esfingosina (Figura 3) pueden variar en la longitud de la cadena alquílica (de 14 a 22 átomos de carbono), en el grado de saturación, en la posición de los dobles enlaces, en la presencia de un grupo hidroxilo

en posición 4 y en la ramificación de la cadena alquílica para formar diferentes esfingolípidos[12, 13]. Una esfingosina suele estar acilada con un ácido graso de cadena larga (14-30 átomos de carbono), por su grupo amino en posición 2 para formar una ceramida.

Las estructuras más usuales de esfingolípidos son ceramidas con diferentes sustituyentes polares en el grupo hidroxilo del C1.

Hasta la fecha han sido encontrados más de 300 esfingolípidos con cabezas polares diferentes. Entre estos compuestos se encuentran la esfingomielina (SM), los cerebrósidos y los cerebrosulfátidos que abarcan las tres grandes categorías de esfingolípidos que conocemos hoy (fosfoesfingolípidos y glicosfingolípidos ácidos y neutros) [13, 14].

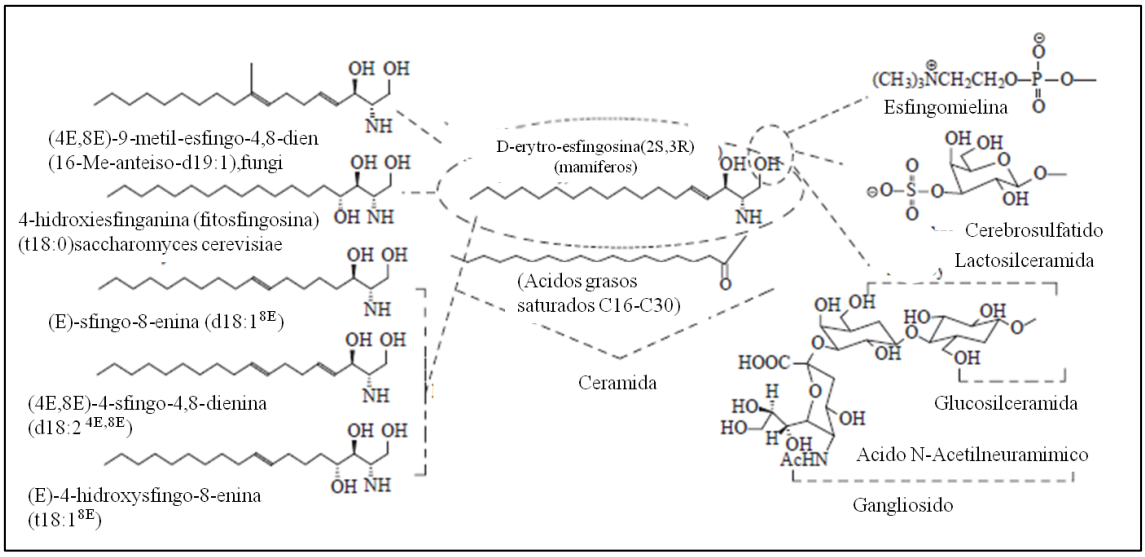


Figura 3. Estructura química de esfingolípidos [10].

Éter fosfolípidos

Los éter fosfolípidos contienen grupos alquilo o alquilo-enil [14]. Los éter fosfolípidos más importantes en el organismo se denominan plasmalógenos, los cuales poseen el O-alkil-1-enil con un doble enlace cis adyacente al enlace éter.

Fosfonolípidos

La distribución de fosfonolípidos en la naturaleza se limita principalmente a ciertos animales inferiores, tales como los moluscos, protozoos y bacterias, por tanto, tienen menor relevancia para la salud humana. Son residuos del ácido 2-Aminoetillfosfónico (ciliatina) unidos a una ceramida o a un diacilglicerol [15, 16].

5.1.3 Propiedad anfifílica de los fosfolípidos

Los PLs poseen un extremo hidrófilo y otro hidrófobo, lo que les aporta carácter anfifílico. Esto explica su comportamiento cuando se encuentran en medio acuoso, siendo capaces de autoasociarse eliminando el contacto energéticamente desfavorable entre la parte hidrófoba de estas moléculas y el agua. De la misma manera colocan su parte hidrófila en contacto con el agua. Esta auto-asociación de PLs da lugar a agregados moleculares de muy diferente tamaño y geometría. Los agregados se organizan de forma diferente según sea su balance hidrofílico/lipofílico, grado de ionización o concentración, dependiendo también de factores externos como temperatura, pH o fuerza iónica. La Figura 4 representa algunas de las estructuras básicas encontradas con frecuencia cuando los PLs se mezclan en solución acuosa, tales como micelas o liposomas. Además, en presencia de disolventes orgánicos, los PLs tienden a formar micelas inversas [17, 18].

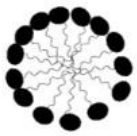
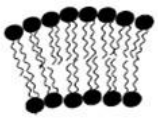
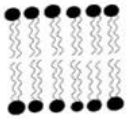
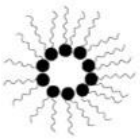
Forma	Organización	Fase	Tipo
		Micelas Hexagonal	Cadena simple con cabeza polar grande
		Laminar flexible (vesícula)	Cadena simple con cabeza polar pequeña o cadena doble con cabeza polar grande
		Laminar cubica	Cadena doble con cabeza polar pequeña
		Micelas inversas Hexagonal	Cadena doble poliinsaturada con cabeza polar pequeña no iónica.

Figura 4. Estructuras básicas formadas por los PLs [17]

La propiedad anfifílica le otorga a los PLs su gran capacidad emulsionante, sin duda los PLs son considerados importantes ingredientes tecnológicos, cuyo uso principal es la formación de emulsiones. Las emulsiones son termodinámicamente inestables y tienden a volver a transformase al sistema de fases separadas del que provienen. Sin embargo, tras la adición de emulsionantes adecuados, tales como PLs, las emulsiones se vuelven cinéticamente estables durante un periodo de tiempo más duradero [17]. Como los PLs contienen tanto un grupo hidrófilo como un grupo hidrófobo, se pueden orientar en la interfase aceite-agua reduciendo la tensión interfacial entre las dos fases. De este modo, disminuye la tendencia de la coalescencia, que es el proceso en el que dos gotas con la misma composición entran en contacto para formar una gota de mayor tamaño. Pero además del agente emulsionante, la estabilidad de las emulsiones depende de otros factores como la viscosidad de las fases y la relación de volumen de fases [19].

Mención especial merece la formación de liposomas, que también son consecuencia del carácter anfifílico de PLs. Cuando los PLs se dispersan en medios acuosos se

pueden formar espontáneamente vesículas esféricas, estas vesículas comúnmente conocidas como liposomas contienen membranas compuestas por PLs. La estructura de los liposomas da lugar a la separación de dos fases acuosas por una membrana. Estos liposomas se pueden utilizar para atrapar distintas moléculas dentro del compartimento acuoso central si son solubles en agua. Además, si las moléculas son de naturaleza apolar, se pueden integrar dentro de la membrana. A diferencia de la micela, en un liposoma las cadenas hidrocarbonadas apolares se agrupan formando capas, de modo que crean esferas bicapa concéntricas que envuelven por completo una región interna de agua. Los primeros liposomas, fueron descritos en 1965 y pronto se propusieron como sistemas de administración de fármacos. El trabajo científico durante casi 5 décadas ha conducido al desarrollo de avances técnicos importantes para utilizar los liposomas en diversas aplicaciones, tales como, carga de fármacos, extrusión con tamaño homogéneo, liposomas de liberación, liposomas que contienen polímeros de ácidos nucleicos, liposomas de ligando específico y liposomas que incorporan combinaciones de fármacos. Estos avances han dado lugar a numerosos ensayos clínicos en áreas tan diversas como cáncer, antibióticos, anestésicos o antiinflamatorios [20, 21]. Por otro lado, en la actualidad los liposomas también son utilizados en cosméticos debido a sus efectos hidratantes. La aplicación actual más relevante en relación a la tecnología alimentaria, es su utilización como lípidos portadores que mejoren el transporte de compuestos bioactivos.

Los liposomas se clasifican generalmente de acuerdo a su rango de tamaño, número de láminas y volumen interno disponible. En la Figura 5 se presenta un esquema con ilustraciones de liposomas de diferente tamaño y número de láminas. Se puede distinguir entre vesículas multilamelares (MLV), vesículas multivesiculares (MVV), vesículas unilamelares grandes (LUV), o vesículas unilaminares pequeñas (SUV). Mientras que los liposomas SUV muestran un diámetro de aproximadamente 20 a 100 nm, los LUV, MLV, y MVV tienen un tamaño desde un pocos cientos de nanómetros a varios micrones [17].

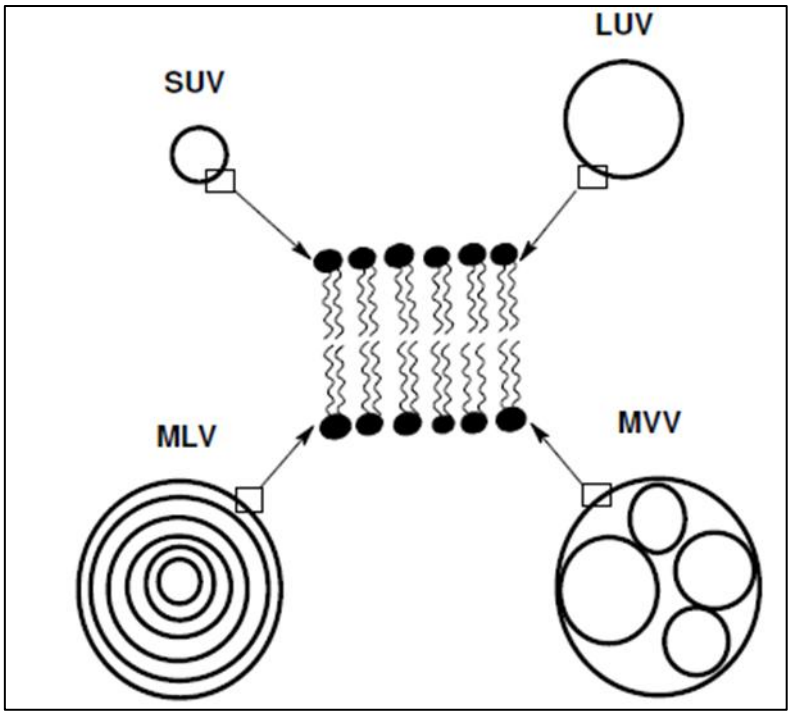


Figura 5. Representación de los principales tipos de liposomas. [17]

5.1.4 Fosfolípidos en la dieta

Los PLs han adquirido una gran importancia comercial por sus reconocidos beneficios para la salud. Sin embargo, la creciente demanda de productos alimenticios bajos en grasa, conlleva generalmente una reducción del consumo de PLs. La ingesta normal de PL es de 2-8 gramos por día, lo que representa del 1 al 10% de la ingesta total de grasa al día. Huevos, casquería, carnes magras, pescado, mariscos, granos de cereales y semillas oleaginosas son alimentos con un alto contenido en PLs. Sin embargo, hortalizas, frutas y tubérculos, contienen niveles relativamente bajos [22]. En la Tabla 1 se muestran las principales fuentes de PLs. El fosfolípido más común en los alimentos es la PC y suele comprender el 90% de los PLs de la dieta, aunque también está disponible como suplemento alimenticio en un producto conocido como lecitina. Otros PLs, tales como la PE, PS y PI están presentes en cantidades mucho más pequeñas en la dieta. PE es el segundo fosfolípido más abundante, se encuentra sobretodo en hígado y cerebro, aunque también está presente en plantas. El PI es abundante en tejido cerebral y en menor cantidad en productos como la soja. En cuanto a la SM, está

presente en huevos, carne y pescado y se ingiere en un nivel de 0,3-0,4 gramos por día [22].

Tabla 1. Contenido de lípidos totales y PLs en algunos alimentos.

	Lípidos totales (g / 100g alimento)	mg/100g						
		Total PL	PC	PE	P1	PS	SM	LPC
Yema de huevo	31.8	10306	6.771	1.917	64	-	486	419
Hígado de cerdo	3.7	2901	1688	618	209	38	131	61
Hígado de pollo	5.6	2542	1120	829	-	146	291	-
Soja	20.8	2308	917	536	287	-	-	-
Calamar	1.68	1098	777	114	-	83	102	-
Pechuga de pollo	1.12	783	391	187	-	100	56	-
Carne de ternera	4.1	660	407	207	-	-	46	-
Cacahuete	48.5	620	270	50	150	-	-	--
Bacalao	2.2	580	331	128	23	29	29	6
Espinaca	0.3	157	37	36	11	-	-	-
Patata	0.15	76	38	22	12	1	-	-
Zanahoria	0.3	55	23	15	5	3	-	-
Manzana	0.09	40	21	10	6	1	-	-
Leche de vaca	3.7	34	12	10	2	1	9	-

5.1.5 Suplementos alimenticios

El consumo de PLs como suplementos alimenticios ha aumentado significativamente en los últimos años, por lo que existe una gran cantidad de productos comerciales disponibles (Tabla 2).

Concretamente en el campo de la salud humana, los PLs están indicados en la prevención de diversas enfermedades como hipercolesterolemia, aterosclerosis y enfermedad cardiovascular por la capacidad de inhibir la absorción de colesterol [23, 24]. En cuanto al control de la obesidad, diversos estudios han demostrado la capacidad del PI para disminuir los lípidos en plasma e hígado, además, también se ha

detallado la prevención de enfermedades relacionadas con obesidad, proporcionada por fosfatidilcolina conteniendo AGs omega 3 de cadena larga [25, 26]. Respecto al desarrollo cognitivo y cerebral, la PS ha mostrado mejoras en propiedades cognitivas y resultados positivos en Alzheimer, pérdida de memoria, estrés y depresión [27]. Como mediadores de inflamación e inmunidad, los derivados oxidados de los PLs tienen una importante implicación en la activación de la inflamación. Por otro lado, los lisofosfolípidos están implicados en la regulación de la respuesta inmune [28, 29].

Tabla 2. Suplementos de base fosfolipídica disponibles comercialmente.

[30].

Nombre producto	Información del producto
	Procedentes de Soja
Essentiale ®	80% PC. Otros ingredientes: aceite de soja, aceite de ricino, etanol, etil-vainillina, metoxiacetofenon, colorantes alimentarios, gelatina, tocoferol y el agua; en forma de cápsulas. Nattermann & Cie GmbH
Essentiale L ® o Buer ® Lecitina	Fosfolípidos soja (pureza desconocida), otros ingredientes: riboflavina, fosfato de sodio, clorhidrato de piridoxina, cianocobalamina, pantotenato de sodio y nicotinamida, en forma de cápsulas. Nicholas Piramal India Ltd. O Nycomed GmbH Alemania
S45 Lipoide ®	45-50% de PC de soja, 10-18% PE, máx. 4% LysoPC. Composición ácidos grasos: ácido linoleico 58-65%, 12-17% ácido palmítico, ácido oleico 8-12% y otras AF; como compuesto en polvo. Lipoid GmbH
Lipostabil ®	93% de PC. Otros ingredientes: etanol, alcohol bencílico, ácido desoxicólico, hidróxido de sodio, cloruro de sodio, tocoferol y agua; como formulación líquida. Artegodan GmbH
PC-55	55% PC, 30% PE, PI 3%, otras PLs y triglicéridos; compuesto en polvo. Twinlab
PhosChol	80% de lecitina de soja (Phosal 75A), 18% de TG. Otros ingredientes: anetol, tocoferol y parabenos. Composición de ácidos grasos: ácido linoleico, principalmente; como formulación líquida. Nutrasal LLC Co.
Phospholipon ® 100	100% PC de soja como compuesto en polvo. fosfolípidos GmbH
Phospholipon 90G ®	94-100% de PC, LysoPC y tocoferol; compuesto en polvo. GmbH
PC poliinsaturada	PC con ácido linoleico 40-52% (n-6); como compuesto en polvo. Rhône-Poulenc Rorer GmbH
Lecitina poliinsaturada	60% de PC, 30% de PE, ácido fosfatídico 6%, 3% monophosphatidylinositol, 3% LysoPC, los ácidos grasos insaturados de 80%, 20% de otros ácidos grasos; compuesto en polvo. American Lecithin Co.
Lecitina de soja	31.7% PC, 20,8% de PE, PS 3%, 17,5% PI, ácido fosfatídico 2%, otros

	ingredientes desconocidos; compuesto en polvo. Herbario, Brasil
Lecitina de soJa	40.4% PC, 35,1% de PE, 24,5% PI. Composición de ácidos grasos: 56,6% de linoleato, 18,9% de palmitato y otros ácidos grasos; compuesto en polvo. Central Soya, Fort Wayne IN
Soja PC	96,5% PC como compuesto en polvo. Tsujiseiyu Matsuzaka Co. [
Cápsulas de PC de soja	64% de PC de soja, 30% de aceite de soja, otros PLs, etanol y agua. Composición de ácidos grasos: ácido 64% linoleico, ácido palmítico 12%, ácido oleico 12%, ácido linolénico 8% y ácido esteárico 4%; como compuesto líquido. Nutrition et Santé, Revel, Francia
Sterpur P-30	30% de PC, 21% de PE y 8% de PI, otros ingredientes desconocidos; como granulado. Stern-Lecitina de Soja y GmbH
	Procedente de leche
Lacprodan ® PL-20	27% PC, 22% PE, PI 8%, 27% SPM, 12% PS y otros ingredientes; compuesto como en polvo. Arla Foods Ingredients.
Esfingomielina	Extraído de la leche bovina, composición desconocida; como compuesto en polvo. Avanti Polar Lipids, Inc.
	De origen marino
MPL (oil Krill Neptune, NKO ™)	40% PL, 15% de EPA y el 9% de DHA obligados a PLs y lípidos neutros, otros ingredientes: insaturada FAS, saturado AF, gelatina, glicerina, agua, como formulación líquida envasada en cápsulas de gelatina. Jarrow Formulas Co.
MPL (Vitalipin ®)	29% PC, 18% de EPA y 26% DHA unido a PLs y lípidos neutros, otros ingredientes: polienos, otras grasas; formulación líquida envasada en cápsulas de gelatina. Membramed GmbH alimentos saludables
PC de calamar	PC contiene ácido palmítico 35,2%, el 9,2% de EPA, DHA 43% y el 13% de otros ácidos grasos; compuesto en polvo. Nippon química RSS Co. Ltd.
	De origen animal
PI de hígado bovino	100% PI como compuesto en polvo. Avanti Polar Lipids, Inc.
PS derivados de cortez cerebral	Composición desconocida. Fidia Farmaceutical S.p.A.
GPLs de cerebro de cerdo	PLs con aprox. 23% ácidos grasos saturados, 53% de ácidos grasos monoinsaturados y 24% de ácidos grasos n-6, como compuesto en polvo. Laboratoires Ponroy, Francia

.

5.1.6 Aplicación tecnológica de fosfolípidos

Además de su actividad biológica, los PLs poseen numerosas aplicaciones tecnológicas en la industria de la alimentación, farmacia y cosmética [4, 31]. Los PLs han sido ampliamente utilizados como aditivos tecnológicos; estabilizadores, texturizantes, dispersantes, emulsionantes o antioxidantes. Debido a sus propiedades anfifílicas, los PLs obtenidos de subproductos del aceite, leche y huevo, son utilizados sobretodo como tensioactivos y emulsionantes en la industria alimentaria, principalmente bajo el nombre de lecitina [32].

Ciertos PLs especiales están diseñados como prodrogas para controlar la liberación o mejorar la absorción de fármacos. La industria farmacéutica utiliza diversos tipos de PLs en la formulación de liposomas, como por ejemplo los formados con dimiristoil-PC, que poseen efectos terapéuticos en metástasis. En medicina, muchos PLs son marcados y utilizados como herramienta para estudiar procesos bioquímicos. El estudio del perfil de PLs por resonancia magnética nuclear es empleado en el diagnóstico de tumores cancerígenos [33, 34]. Por otro lado, la PC se emplea como un agente tensioactivo pulmonar en el tratamiento de la dificultad respiratoria neonatal ya que los PLs comprenden el 80% de los surfactantes pulmonares [35]. Los PLs también tienen aplicación como aditivos en otros sectores industriales, en pinturas, plástico, caucho, vidrio, cerámica, productos asfálticos, industria del petróleo, procesamiento de metales, pesticidas, adhesivos, textiles y cueros [32].

5.1.7 Lecitina

La lecitina es la designación dada a un conjunto de triglicéridos (TGs) y PLs, (mayoritariamente PC) con diferentes grados de pureza. Sin embargo, en bioquímica, el término lecitina es usualmente utilizado como sinónimo de PC pura.

La elaboración de la lecitina de origen vegetal se basa en la extracción de PLs de aceites mediante un proceso de desgomado. Tan solo los PLs hidratables son los que constituyen el producto comercial llamado lecitina, ya que las gomas hidratadas obtenidas se extraen y se secan al vacío. Los fosfolipidos no hidratables como el PA y el ácido lisofosfatídico contenidos en el aceite no forman parte de la lecitina [36]. De

hecho, tras los últimos estudios, parece que PC y PI son hidratables en todos los casos, sin embargo PE y PA pueden ser hidratables o no dependiendo de su capacidad para formar sales. En cuanto al ácido lisofosfatídico, no todas sus sales son hidratables [37].

Las semillas de girasol se pueden considerar una fuente potencial de lecitina, debido a su contenido en PLs [38], pero la fuente más habitual para la obtención de lecitina es el aceite de soja. Por otro lado, también la lecitina procedente de yema de huevo está disponible comercialmente. Las diferentes lecitinas tienen distintos usos y aplicaciones, así, por ejemplo, la lecitina de huevo es más resistente a la oxidación, y la de soja da lugar a emulsiones más estables [39].

La lecitina de soja se comercializa principalmente como suplemento alimenticio. El contenido aproximado en PLs de la lecitina es: PC 20%, PE 15%, PI 20%, otros fosfátidos 5%, carbohidratos y esteroles 5%, y otros glicéridos el 35% [40, 41]. Además, la lecitina, se utiliza ampliamente como aditivo alimentario, y debido a sus propiedades emulsionantes, también es empleada como aditivo en la fabricación de productos cosméticos, farmacéuticos y de alimentación animal, [41, 42]. La lecitina es un ingrediente estándar en la margarina, proporciona consistencia y textura en salsas y otros productos cremosos. Actúa como emulsionante en la fabricación de productos lácteos como chocolate, quesos o helados, y ayuda a la dispersión de alimentos en polvo como leche y cacao [43, 44]. La lecitina también se utiliza con frecuencia como agente antiagregante en panadería y confitería para formar complejos con el almidón mejorando el ablandamiento de la miga y prolongando la vida útil del pan [45, 46].

5.2 INGREDIENTE FUNCIONAL

La sociedad está adquiriendo cada vez más conciencia de los problemas asociados a dietas con ciertas deficiencias en algunos nutrientes. En el pasado, los alimentos sólo eran valorados por su sabor, aroma o valor nutricional en general, pero actualmente, la mayoría de consumidores reconoce que existen categorías adicionales relacionadas con los alimentos. Este es el caso de los alimentos funcionales, que además de proporcionar las necesidades energéticas y nutricionales básicas, son capaces de aportar beneficios adicionales a nuestra salud.

Los consumidores se sienten cada vez más atraídos por estos productos buscando principalmente prevenir la aparición de ciertas enfermedades crónicas. Hay dos razones primordiales por las que el consumidor se puede sentir atraído por estos productos: 1) la falta de estudios científicos en algunos remedios naturales y tradicionales y 2) el alto coste de los medicamentos.

La primera vez que se contemplaron alimentos procesados que contenían ingredientes con un posible efecto fisiológico beneficioso fue en Japón en 1991, y dio lugar a la aparición del término FOSHU (acrónimo en inglés de Food Of Specific Health Use) [47].

Actualmente, una gran cantidad de compuestos bioactivos son introducidos en diversos alimentos para aportarles la capacidad de prevenir cierto tipo de enfermedades, apareciendo así el concepto de ingredientes funcionales. Los compuestos bioactivos pueden ser incorporados de diferentes formas en la matriz alimentaria como emulsiones, microemulsiones, soluciones micelares y suspensiones lipídicas, pero pretendiendo que el nuevo ingrediente esté incluido de forma homogénea y estable. Al incorporar estos compuestos en diferentes matrices de diversa naturaleza, han surgido principalmente dos necesidades, la capacidad de los procesos tecnológicos de incorporar los compuestos en la matriz deseada y la capacidad del organismo de absorber estos compuestos dependiendo de la forma en la que son introducidos. En la búsqueda de la solución a estos problemas, surge el concepto de lípidos portadores, los cuales, pueden conseguir la liberación eficaz de un compuesto bioactivo y pueden optimizar tanto la extensión como la velocidad de absorción de dichos compuestos. Esta estrategia fue desarrollada principalmente en el campo farmacéutico, pero puede ser extendida a los campos de nutracéuticos y alimentos funcionales. Estos vehículos lipídicos pueden aportar beneficios adicionales al ser incorporados en alimentos, protegiendo nutrientes y otros componentes bioactivos y mejorando su bioaccesibilidad durante la etapa de digestión de los alimentos. De este modo, es posible reducir pérdidas del componente activo o evitar interacciones con inhibidores de absorción. Además, estos vehículos lipídicos podrían mejorar el transporte de moléculas bioactivas a diferentes tejidos y la biodisponibilidad oral de compuestos poco solubles en agua.

5.3 LÍPIDOS PORTADORES DE COMPUESTOS BIOACTIVOS

En la industria farmacéutica, el uso de formulaciones basadas en lípidos es uno de los varios enfoques aplicados para mejorar la biodisponibilidad de compuestos poco solubles en agua. En el caso específico de los sistemas de lípidos portadores para la administración de fármacos, se incluyen dos grupos, los sistemas lipídicos naturales como TGs, diglicéridos (DGs), PLs, AGs o esteroles, y los sistemas lipídicos sintéticos o también llamados lípidos estructurados. Estos lípidos estructurados son lípidos portadores de diseño modificados para conseguir mejorar la bioaccesibilidad y la biodisponibilidad de compuestos bioactivos. En este caso, en lugar de incorporar un compuesto bioactivo junto con el lípido portador, el compuesto es introducido en la estructura molecular del lípido.

Inicialmente, los sistemas lipídicos de administración de fármacos demostraron ser eficientes y por ello, han recibido mucha atención por parte del ámbito académico. Así podemos distinguir diferentes sistemas basados en lípidos para la administración de fármacos como, emulsiones, microemulsiones, soluciones micelares, suspensiones lipídicas y sistemas autoemulsionables (SEDDS) [48, 49].

Los SEDDS menos conocidos, pero muy interesantes, son mezclas isotrópicas que pueden contener lípidos, surfactantes, solventes y cosolventes, y que son usados en el diseño de formulaciones para mejorar la absorción de compuestos lipófilos. Estos SEDDS forman emulsiones de aceite en agua relativamente estables en contacto con los fluidos gastrointestinales, por acción del peristaltismo [49, 50]. A modo de ejemplo, la biodisponibilidad de la vitamina E (poco soluble en agua) ha sido estudiada en diferentes sistemas como puede ser su disolución en aceite de soja. Julianto y colaboradores demostraron que un sistema formado por monoleato de sorbitol, monoleato de sorbitol polioxietilinado (polisorbato) y tocoferol disuelto en aceite de palma, en las proporciones 4:2:4, aumenta los niveles en plasma de vitamina E entre un 200-400% comparando los niveles plasmáticos de dicha vitamina administrada en una cápsula de gelatina [51].

5.3.1 Lípidos estructurados portadores

Los lípidos estructurados son acilgliceroles, PLs y otros lípidos cuya estructura ha sido modificada selectivamente por métodos químicos y/o enzimáticos. Dichas modificaciones incluyen la incorporación de nuevos AGs y otras moléculas, así como la reestructuración de los AGs que los componen con el fin de producir nuevos lípidos con mejores funcionalidades. La síntesis de estos lípidos portadores conduce a la "lipofilización" (lipophilization) de compuestos bioactivos hidrófilos, modificando el equilibrio hidrófilo/lipófilo de tales moléculas, lo que mejora su bioaccesibilidad y su biodisponibilidad. Ello permite, por ejemplo, ampliar la utilidad de antioxidantes bioactivos cuyo empleo está muchas veces limitado por su difícil solubilización en diversas matrices. Por otro lado estos lípidos portadores pueden dirigir las moléculas bioactivas a órganos o tejidos específicos. Es importante destacar que algunos lípidos portadores pueden ser por sí mismos compuestos biológicamente activos y se pueden producir acciones sinérgicas con la sustancia bioactiva que vehiculizan.

5.3.2 Lípidos estructurados en la industria farmacéutica.

La utilización de lípidos estructurados en la industria farmacéutica, persigue los siguientes objetivos: mejorar la biodisponibilidad, regular la excreción, reducir la toxicidad o superar problemas en la formulación de distintas sustancias bioactivas. Por ejemplo, se han producido antiinflamatorios no esteroideos como ácido acetil salicílico, naproxeno, indometacina e ibuprofeno unidos a glicéridos con el fin de reducir su ulcerogenicidad [52]. Otros fármacos han sido modificados con el fin de promover su difusión a través de la barrera hematoencefálica, como por ejemplo el neurotransmisor GABA, glicina o L-Dopa unidos a glicéridos [52-54].

En el caso específico de los PLs estructurados en la industria farmacéutica, se pueden distinguir dos casos de modificaciones sobre el fosfolípido: la sustancia bioactiva unida a la cabeza polar del fosfolípido a través del grupo fosfórico, o su unión en una de las dos posiciones de la molécula de glicerina. Así por ejemplo, es bien conocida la adicción de nucleósidos por su actividad antiviral o antineoplásica a la cabeza polar de fosfolípidos [52, 55, 56]. De esta forma, estos nucleósidos tienen una mejor absorción

y comportamiento farmacocinético. Como ejemplo de PLs modificados por reemplazo de AGs, se ha descrito la síntesis de ácido valproico e ibuprofeno unidos a PLs [57].

5.3.3 Lípidos estructurados en la industria alimentaria.

En el campo de la tecnología de alimentos, son muchas las moléculas bioactivas que tienen baja solubilidad en agua, lo que dificulta su manipulación e inclusión en determinados alimentos. Además, esta limitación puede disminuir la funcionalidad de tales moléculas, ya que la solubilización luminal y la disolución en el tracto gastrointestinal, son eventos necesarios para su biodisponibilidad [58, 59]. De esta forma los lípidos portadores estructurados que funcionan como sistemas de administración de ingredientes alimentarios bioactivos, mejoran su incorporación a matrices alimentarias, su biodisponibilidad o su actividad biológica. Numerosas moléculas bioactivas aparecen continuamente en la literatura científica reclamando su uso potencial como ingredientes funcionales o nutracéuticos, tales como ácidos fenólicos, esteroles, AGs o vitaminas. Un caso muy estudiado, es la adicción de antioxidantes naturales en alimentos funcionales. En muchos casos el antioxidante presenta baja solubilidad en agua o en aceite, lo que afecta y dificulta su aplicación tecnológica en diversos alimentos. En este sentido, la obtención de lípidos estructurados que incorporan antioxidantes como ácido ascórbico o compuestos fenólicos en su estructura, ha sido ampliamente estudiada [60-62].

Los PLs estructurados son un caso especial, debido al carácter anfifílico y a la mayor complejidad de su estructura, principalmente por su cabeza polar. Los PLs por tanto, tienen una diferente capacidad para incorporar compuestos bioactivos en comparación con TGs, DGs y monoglicéridos [63, 64]. Los PLs estructurados modificados en su composición de AGs son sintetizados enzimáticamente para incorporar AGs bioactivos de cadena corta, media o larga. La incorporación en PLs del ácido hexanoico, linoleico conjugado (CLA), o poliinsaturados n-3 como los ácidos EPA y DHA [8] han sido extensamente estudiados [65-68].

Por otro lado, diversos antioxidantes como ácido ascórbico, tocoferol y compuestos fenólicos han sido incorporados también en la molécula del fosfolípido, modificando su

cabeza polar [31, 64]. La mayor complejidad encontrada en la síntesis de PLs estructurados, es la elección de enzimas y medios de reacción adecuados, teniendo en cuenta la naturaleza química de los sustratos de reacción, ya que su polaridad y solubilidad condiciona en gran medida el progreso de estos bioprocesos.

5.3.4 Fenolípidos

Los compuestos fenólicos se encuentran prácticamente en todas las plantas de forma natural y son compuestos habituales en la dieta humana. Por otro lado, han despertado un gran interés por su potencial como antioxidantes naturales en alimentos procesados. Los compuestos fenólicos poseen uno o más anillos aromáticos con uno o más grupos hidroxilo y se clasifican en: flavonoides, ácidos fenólicos, taninos, estilbenos y lignanos; siendo estos dos últimos menos comunes. Los compuestos fenólicos, son los metabolitos secundarios más abundantes de las plantas, con más de 8.000 estructuras fenólicas actualmente conocidas, que van desde moléculas simples, tales como ácidos fenólicos, a sustancias altamente polimerizadas como taninos.[69]

Los flavonoides constituyen el mayor grupo de compuestos fenólicos de las plantas, lo que representa más de la mitad de los ocho mil compuestos fenólicos naturales. Las variaciones en la estructura de estos compuestos dan lugar a las clases principales de flavonoides, es decir, flavonoles, flavonas, flavanonas, flavanoles, isoflavonas, y antocianidinas. De manera similar a los flavonoides, los ácidos fenólicos constituyen un importante número de compuestos fenólicos con funciones bioactivas [70]. En los últimos años, se ha prestado gran atención a los compuestos fenólicos bioactivos debido a su capacidad para promover beneficios para la salud humana, reduciendo la incidencia de diversas enfermedades [71, 72]. Existen numerosos estudios que atribuyen a los polifenoles de la dieta un importante papel en la prevención de cáncer, osteoporosis, diabetes mellitus, enfermedades neurodegenerativas y enfermedades cardiovasculares [73, 74]. Además, a los compuestos fenólicos se les ha atribuido capacidad antioxidante, limitando el riesgo de padecer enfermedades asociadas al estrés oxidativo, ya que pueden proteger estructuras celulares del ataque de especies

reactivas del oxígeno. Por otro lado, en las últimas décadas se han descrito otros muchos mecanismos en los que participan estos compuestos. Entre estos mecanismos destacan sus capacidad para modular la actividad de enzimas tales como la telomerasa [75], la ciclooxigenasa [76], la lipooxigenasa [77], o su capacidad para interaccionar con señales de transducción y receptores celulares [78, 79]. También poseen capacidad anti-mutagénica, antialergénica y antimicrobiana [80-82]. Debido a estas numerosas características beneficiosas para la salud humana, las investigaciones se han intensificado con el objetivo de encontrar frutas, verduras, plantas y subproductos agroindustriales como fuente de compuestos fenólicos bioactivos [70].

Algunos de los compuestos fenólicos más conocidos en alimentación son el tirosol, el hidroxitirosol, la quercetina, el resveratrol, las isoflavonas y las procianidinas, los cuales pueden encontrarse en la oliva, el vino, la soja, la uva, el té y otras frutas y verduras.

El tirosol y el hidroxitirosol (HT), mostrados en la Figura 6, son compuestos fenólicos destacados en el aceite de oliva virgen que están presentes en la oliva como derivados de secoiridoides o en forma libre. Los secoiridoides son compuestos glicosilados que se producen a partir del metabolismo secundario de terpenos [83].

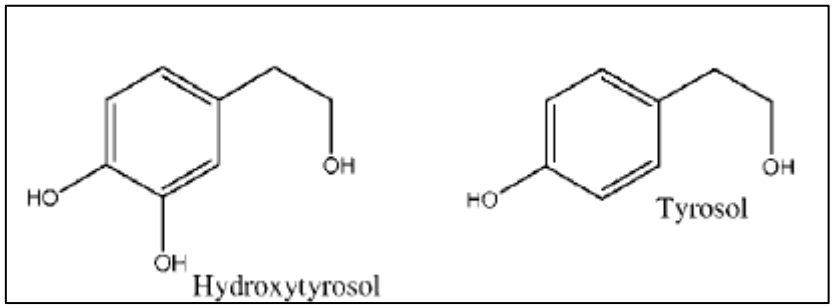


Figura 6. Estructura de tirosol e hidroxitirosol

Aunque factores como la variedad, la madurez de la aceituna, el procesamiento del aceite de oliva o incluso factores agronómicos son fuertes determinantes en la cantidad final de los compuestos fenólicos en el aceite de oliva virgen, se han

cuantificado concentraciones de entre 100 y 600 mg/kg, de los cuales aproximadamente la mitad de esta cantidad corresponden a HT y sus derivados [83]

El HT particularmente, surge de la oleuropeína (éster de HT y ácido elenólico), la oleuropeína, mostrada en la Figura 7, es el componente fenólico mayoritario de la pulpa de las aceitunas verdes de la variedad *Olea europea*.[84]

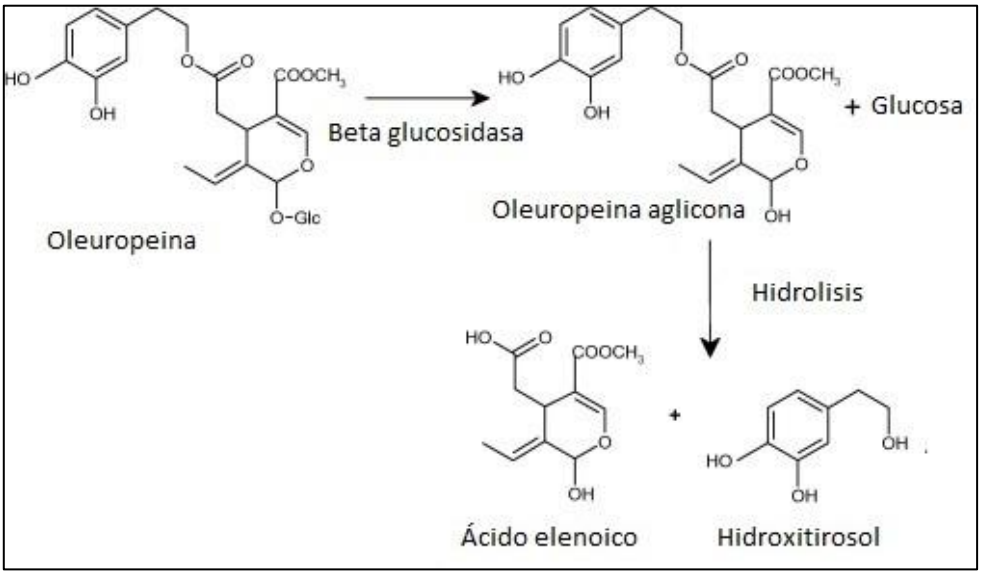


Figura 7. Representación de la formación de hidroxitirosol a partir de oleuropeína

Al igual que otros fenoles, el HT también, ha sido señalado como responsable de los beneficios para la salud asociados al consumo habitual de aceite de oliva virgen. Además, se ha indicado su actividad contra el estrés oxidativo celular, reduciendo los riesgos relacionados con la patogénesis y la proliferación de cáncer [85]. En el mismo sentido, el tirosol posee capacidad protectora de células Caco -2 contra efectos citotóxicos/apoptóticos de LDL oxidada, inhibe la actividad de la 5-lipoxigenasa de leucocitos, y mejora los sistemas de defensa antioxidante intracelulares. Aunque, el tirosol es un compuesto fenólico que no posee una marcada actividad antioxidante, se ha demostrado que ejerce potentes efectos protectores contra las lesiones oxidativas celulares reforzando otras defensas antioxidantes intracelulares [86].

Sin embargo, debe tenerse en cuenta que la eficacia de tirosol e HT en algunos sistemas biológicos está relacionada con su capacidad de penetrar en las células y ambos tienen baja solubilidad y estabilidad en un entorno lipófilo [87]. La baja

solubilidad de los compuestos fenólicos en medios hidrófobos es un factor clave que limita su aplicación y biodisponibilidad [88, 89]. La modificación de estos compuestos fenólicos ha sido descrita anteriormente, su incorporación en la estructura de un lípido da lugar a nuevas moléculas con un equilibrio hidrófilo/lipófilo modificado. Mediante estas transformaciones catalizadas usualmente por lipasas, se pueden utilizar compuestos fenólicos con propiedades antioxidantes en matrices lipídicas [58]. Aunque la biodisponibilidad del HT no parece especialmente problemática, recientemente se ha demostrado que sus ésteres sintéticos son mejor absorbidos que el HT sin modificar [90]. Otra de las razones del interés en la producción de derivados lipídicos de HT, es la mejora de su empleo como antioxidante en alimentos. Por esta razón, se han estudiado derivados de HT con mejor equilibrio hidrófilo/lipófilo, para su posible utilización como antioxidantes en alimentos, al mismo tiempo que se intenta mejorar la biodisponibilidad del HT. Una metodología descrita para convertir el HT en una molécula más lipófila, es la esterificación del alcohol primario sin afectar al grupo catecol [91]. Más de 40 análogos lipófilos del HT han sido descritos empleando como biocatalizador la lipasa de *Candida antárctica* [92], en algunos casos, los análogos del HT obtenidos fueron incluso más eficaces que el tocoferol como antioxidantes, mostrando además, efecto protector contra el daño oxidativo en células humanas [91]. Por otro lado, la síntesis de oleato de tirosol catalizada por dos lipasas inmovilizadas para su utilización como ingrediente alimentario, también ha sido descrita. Dicha "lipofilización" del tirosol mejoró ligeramente la actividad antioxidante de este compuesto fenólico en matrices oleosas en comparación con tirosol libre [93].

Además de tirosol e HT, otros muchos compuestos fenólicos han sido objeto de lipofilización. Este es el caso de flavonoides, ya que su actividad biológica se ve limitada por su baja solubilidad. En este sentido, diferentes modificaciones sobre flavonoides han sido descritas, como por ejemplo, la esterificación de diferentes flavonoides con AGs insaturados [94, 95]. La esterificación de AGs con isoflavonas, otorga a las isoflavonas el balance hidrófilo/lipófilo necesario para su incorporación en LDL, incrementando así la resistencia a la oxidación de las LDL [96].

El ácido cafeico y el ácido ferúlico conocidos por sus propiedades antioxidantes tambien han sido modificados para obtener derivados más lipofílicos. [97, 98]. Por otro lado, el ácido rosmarínico, que es un ácido polifenólico que participa en la prevención

de la oxidación de LDL y la inhibición de la actividad de proliferación celular, además de tener actividades antibacterianas, antivirales y antioxidantes, también ha sido transformado en derivados más lipófilos. Los ésteres de ácido rosmarínico formados por procedimientos químicos, han mostrado mayor actividad frente a radicales libres que el ácido rosmarínico [99].

5.4 BIOCATÁLISIS EN LA INDUSTRIA ALIMENTARIA

Los criterios de un desarrollo sostenible que se están implementando en diversos sectores industriales entre los que se encuentra la industria alimentaria, han propulsado el interés por procedimientos ambientalmente más comprometidos. Entre ellos se encuentra la biocatálisis selectiva, aunque todavía es necesario un mayor desarrollo de estos procesos para garantizar su viabilidad a nivel industrial.

El empleo de enzimas como biocatalizadores puede ser considerado como un proceso de “química verde". Las enzimas han sido utilizadas, en múltiples procesos de fermentación y bioconversión en sectores como el farmacéutico, energético, sanitario, alimentario y medio ambiental. Las enzimas presentes en la naturaleza se han utilizado desde tiempos antiguos en la producción de productos alimenticios, tales como queso, masa de pan, cerveza, vino y vinagre, pero en estos procesos se utilizaban microorganismos y no enzimas de forma aislada. El desarrollo de los procesos de fermentación durante la última parte del siglo pasado, destinadas específicamente a la producción de enzimas por la utilización de cepas seleccionadas, hizo posible la fabricación de enzimas purificadas, bien caracterizadas incluso a escala industrial. Este desarrollo ha permitido la introducción de enzimas en los productos y procesos industriales. Las ventajas que los procedimientos biocatalíticos poseen sobre los químicos se basan en que las reacciones catalizadas por enzimas son frecuentemente más regio-, quimio- y estereoselectivas, además de llevarse a cabo en condiciones seguras y muy benignas desde el punto de vista medioambiental. Por otro lado, con los avances en la ingeniería genética, las enzimas pueden sobreexpresarse haciendo los procesos biocatalíticos más económicos y eficientes; además los avances en la modificación estructural de las enzimas permite la creación de nuevas moléculas

proteicas con actividades catalíticas diseñadas a la medida de las necesidades, por ejemplo, la preparación de enzimas termoestables o estables a cierto pH, por medio de la mutagénesis aleatoria o dirigida.

Dos aspectos clave en biocatálisis son el coste del biocatalizador y la necesidad de disolventes para llevar a cabo algunos procesos enzimáticos. Por lo tanto, es de gran interés tanto a nivel académico como industrial, encontrar nuevos medios de reacción ambientalmente benignos y catalizadores eficientes que puedan ser fácilmente reutilizados. En la actualidad, los principales tipos de disolventes limpios son: fluidos supercríticos, disolventes fluorados, líquidos iónicos, agua y por otro lado las reacciones exentas de disolventes. Otra nueva estrategia, es la busqueda de disolventes GRAS (generalmente reconocidos como seguros o Generally Recognized As Safe) [100].

5.5 FOSFOLIPASAS

A grandes rasgos, los problemas encontrados para reemplazar procesos químicos tradicionales por la utilización de fosfolipasas son: las dificultades en el escalado, la separación de producto tras el bioproceso, la tolerancia limitada de las enzimas a determinados sustratos y la necesidad de un conocimiento multidisciplinar para su implantación industrial.

A nivel biológico las fosfolipasas desempeñan un papel fundamental en la regulación celular, metabolismo y biosíntesis de PLs. En concreto, se pueden atribuir a las fosfolipasas tres funciones generales; la función digestiva; el mantenimiento de la membrana: y la regulación de mecanismos celulares y transducción de señales[101]. Por ello, estas enzimas se encuentran en la mayoría de los organismos vivos, incluyendo plantas, animales, hongos y bacterias. Hasta la fecha, cientos de fosfolipasas han sido purificadas, caracterizadas y clonadas [102, 103].

El término fosfolipasas engloba un heterogéneo grupo de enzimas con una gran diversidad de familias, subgrupos e isoenzimas [104, 105]. La clasificación más simple de las fosfolipasas es su distinción según sean acilhidrolasas o fosfodiesterasas. Las

acilhidrolasas incluyen a las fosfolipasas A1, fosfolipasas A2 y fosfolipasas B. Mientras que las fosfodiesterasas engloban a las fosfolipasas C y la fosfolipasas D [101, 106].

Aunque todas las fosfolipasas tienen a los PLs como principales sustratos, se diferencian en la posición del fosfolípido sobre la que actúan, su función, su mecanismo de acción y su regulación. En función de la posición del fosfolípido sobre la que actúan se clasifican como A, B , C y D (Tabla 3 ; Fig. 8). Por ejemplo, la fosfolipasa A1 (PLA1) hidroliza el enlace éster del ácido graso en la posición sn-1 de la molécula de glicerina. De este modo PLA1 y PLA2 producen AGs libres y lisofosfolípidos (LPLs); A su vez, la fosfolipasa B (PLB) rompe el ácido graso unido a LPLs; Por otro lado, Las fosfolipasas C (PLC) escinden el enlace glicerofosfato y las fosfolipasas D tienen capacidad para eliminar el alcohol de la cabeza polar de los PLs. Finalmente las esfingomielasas rompen la SM en fosfocolina y ceramida [107].

La modificación de los AGs de distintos PLs también puede llevarse a cabo en presencia de lipasas. Debido al mayor desarrollo tecnológico y conocimiento teórico y práctico del empleo de lipasas como biocatalizadores, éstas han sido las enzimas más comunes utilizadas para la modificación de PLs.

Table3. Clasificación de fosfolipasas.

Tipo de fosfolipasas	Rotura
PLA1 (EC 3.1.1.32)	Enlace éster entre ácido graso y glicerol en posición sn-1
PLA2 (EC 3.1.1.4	Enlace éster entre ácido graso y glicerol en posición sn-2
PLB (EC 3.1.1.5)(	Enlace éster entre ácido graso y glicerol en posición sn-1 y sn -2
PLC (EC 3.1.4.3)	Primer enlace fosfodiester del glicerofosfolípido
PLD (EC 3.1.4.4)	Segundo enlace fosfodiester del glicerofosfolípido
Esfingomielinasa (EC 3.1.4.1.2)	Fosfodiester de esfingomielina

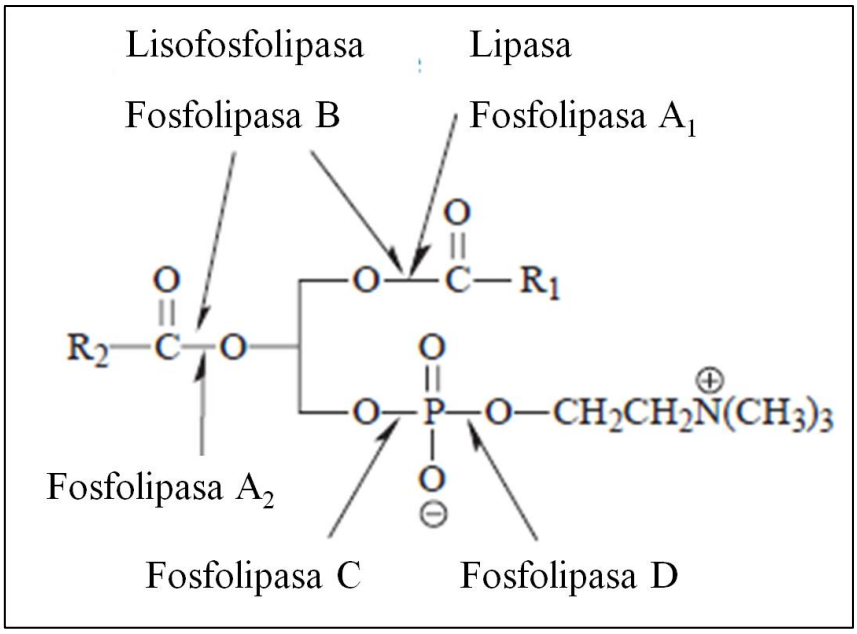


Figura 8. Especifidad posicional de las fosfolipasas. [10]

5.5.1 Aplicaciones de las fosfolipasas

Las fosfolipasas son útiles en aplicaciones industriales ya que proporcionan ciertas ventajas frente a procesos químicos más clásicos. Poseen alta selectividad y pueden actuar sobre un solo compuesto de una mezcla compleja. Requieren condiciones de reacción suaves en comparación con algunas rutas químicas convencionales lo que conlleva a un menor gasto de energía. Además, en ocasiones, los subproductos obtenidos en la producción de enzimas y las propias enzimas son fácilmente biodegradables [100].

Uno de los enfoques más recientes para ampliar el empleo de estas enzimas en biocatálisis es el diseño de fosfolipasas mediante modificación genética de las ya existentes, confiriéndoles propiedades industriales deseables, como especificidad de sustrato, estabilidad y eficiencia [106].

La utilización de fosfolipasas en la producción de alimentos conlleva ventajas atractivas, tales como la mejora de los rendimientos y mayor eficiencia del proceso, ahorro de energía, o una mejora en la calidad del producto final [108]. La industria alimentaria ha incorporado el uso de fosfolipasas en la refinación de aceites comestibles, y en la elaboración de productos lácteos, productos de panadería y otros

productos horneados, productos precocinados y de agentes emulsionantes. Estas fosfolipasas se utilizan con el objetivo de adaptar los PLs para los requisitos específicos de su aplicación, confiriéndoles propiedades funcionales o fisiológicas que la sustancia natural no posee. De este modo, las fosfolipasas se incorporan como adyuvantes en procesos como el desgomado de aceites vegetales durante su refinado, en la fabricación de quesos para la mejora del rendimiento, o en la producción de pan como mejoradores de panificación consiguiendo reducir el uso de agentes emulsionantes [107].

Durante muchos años, el uso comercial de enzimas para la modificación de PLs se restringía al empleo de la fosfolipasa A de páncreas porcino para la obtención de LPLs. Sin embargo, desde finales de la década de 1980 han aparecido un gran número de publicaciones y solicitudes de patente relacionadas con otras enzimas como PLD, PLC y mezclas de fosfolipasas y lipasas, que actúan sobre los PLs [31, 109-111].

Otras aplicaciones de las fosfolipasas además de la industria alimentaria, son la producción de detergentes, la industria cosmética, la industria farmacéutica y el empleo de fosfolipasas como marcadores de diagnóstico de infecciones microbianas.

5.5.2 Fosfolipasa D (EC 3.1.4.4)

Las fosfolipasas D (PLD) están presentes en organismos procariotas y eucariotas, se encuentran en bacterias, hongos, plantas y mamíferos y están involucradas en el metabolismo de PLs, nucleasas y toxinas [112]. La PLD rompe el enlace fósforo-oxígeno entre el fosfato y el alcohol de la cabeza polar de los PLs, liberando ácido fosfatídico (PA) y un alcohol. Esta enzima también puede llevar a cabo reacciones de transfosfatidilación que permiten sustituir el alcohol presente en la cabeza polar del fosfolípido por otro que contenga en su molécula un grupo hidroxilo reactivo. La mayoría de PLDs son capaces de hidrolizar diversos PLs, incluyendo PC, PE, PG, PI, PS, LPC, cardiolipina, y plasmalógenos. [101]

La PLD tiene una posición destacada frente a otra fosfolipasas por su capacidad de transfosfatidilación, por lo tanto, es una de las fosfolipasas más estudiadas en el ámbito científico e industrial junto con las PLAs [113]. Las PLDs han sido aisladas de

diversas fuentes y están disponibles comercialmente. Un caso especial es la PLD de repollo (col rizada), que se puede preparar fácilmente a partir de homogeneizados de este vegetal. También se puede encontrar PLD en otras plantas como la zanahoria, cacahuete, ricino o semillas de algodón. No obstante, las PLDs de plantas se consideran menos apropiadas para la biosíntesis aunque sólo han sido testadas un pequeño número de ellas. Respecto a las PLDs de microorganismos, existen numerosas fuentes. Así, estas enzimas se pueden obtener de diversas cepas de *Streptomyces* y su actividad puede ser caracterizada directamente con métodos espectrofotométricos o mediante medición de la colina formada tras la hidrólisis.

La enzima originaria de *Streptomyces sp.* está disponible comercialmente. Otra PLD producida por un microorganismo, es la producida por la cepa de *Actinomycetes*, que ha sido también estudiada para su empleo en procesos industriales, ya que posee mayor actividad de transfosfatidilación que de hidrólisis [110].

Respecto a la ingeniería genética, diversas PLD de plantas y microorganismos se han obtenido como proteínas recombinantes expresadas en *Escherichia coli*. Actualmente, hay una gran cantidad de entradas relativas a PLD en el NCBI GenBank y ha sido obtenida la estructura terciaria de una PLD de *Streptomyces sp*. como se muestra en la figura 9. Las masas moleculares de PLDs varían entre 16 kDa de *Streptomyces hachijoensis* y 120 kDa de la PLD1 de *Homo sapiens* [64].

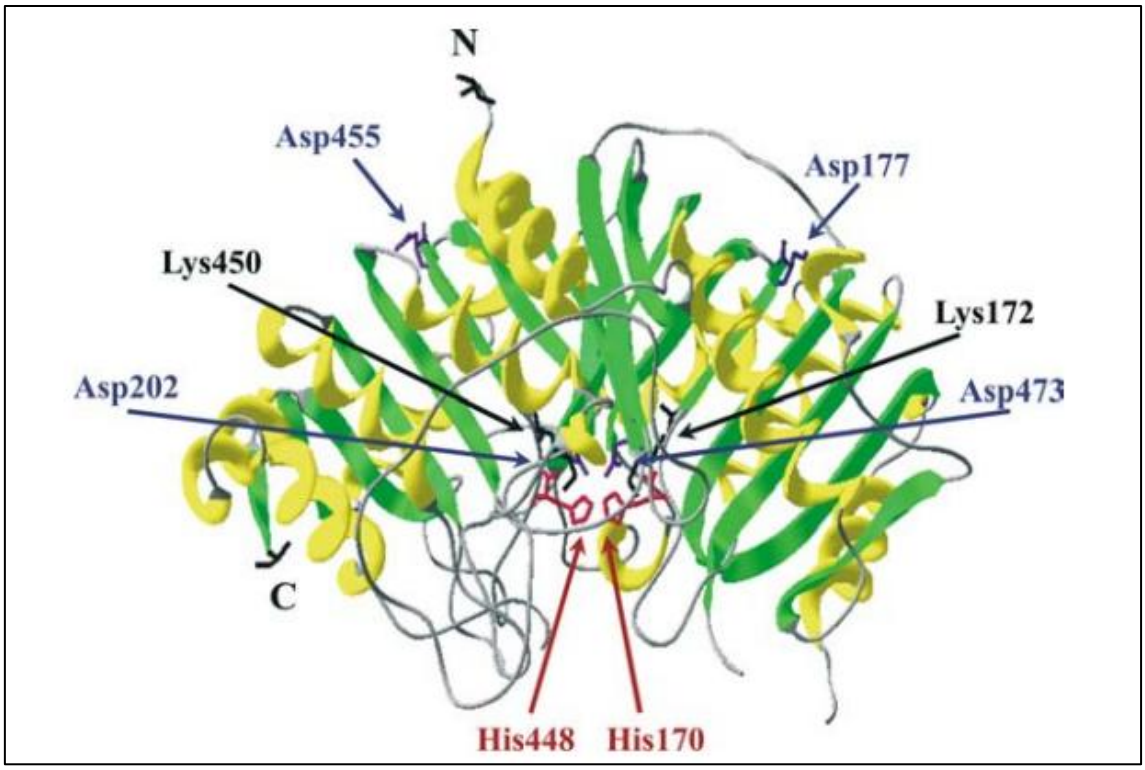


Figura 9. Estructura terciaria de PLD de *Streptomices sp*. Cepa PMF [64]

5.5.3 PLD como adyuvante tecnológico en la industria alimentaria

Diversas fosfolipasas de origen microbiano han sido clonadas y expresadas y dichos resultados han sido recogidos en la literatura científica, e incluso han dado lugar a diversas patentes. Sin embargo, sólo en algunas industrias, tales como panificación, aceite, productos horneados y producción de queso, han sido utilizadas. El objetivo en estos casos, es modificar los PLs que están presentes en el alimento, variar su capacidad emulsionante, o también, eliminar PLs, como en el caso del desgomado de aceites vegetales. Las PLA1 y PLA2 han sido las enzimas más utilizadas desde hace décadas, existiendo diversos preparados comerciales disponibles en el mercado. Sin embargo la fosfolipasa D ha surgido como una nueva opción para mejorar ciertas propiedades reológicas de algunos productos que no es posible conseguir con la utilización de otras enzimas. Este es el caso en la producción de nuevos productos de quesería, panificación o en el tratamiento de yema de huevo, este último, utiliza la

yema tratada como un emulsionante para diferentes alimentos preparados, horneados y salsas.

El empleo de fosfolipasas buscando mejorar la calidad del producto final en la industria láctea es bastante reciente. Así, la PLD se ha probado en la elaboración de productos lácteos con el fin de modificar sus PLs [114]. La utilización conjunta de PLD y PLC se ha propuesto también para la producción del queso utilizado en platos preparados. Una patente reciente describe el tratamiento de la leche con PLC de *Bacillus cereus* y PLD de *Streptomyces chromofuscus* para producir queso mejorando el despegado del molde y aumentando el rendimiento del proceso [115].

La PLD procedente de *Streptomyces chromofuscus* permite mejorar las propiedades reológicas y emulsionantes de yema de huevo. Esta enzima puede ser una alternativa a la aplicación de PLA 2 en la industria alimentaria, debido a que la PLA 2 produce sabor amargo en la yema de huevo por los AGs insaturados liberados. Sin embargo, el tratamiento de la lecitina de yema de huevo con PLD da lugar a colina y PA, evitando así el inconveniente de la aparición de AGs libres. Las emulsiones preparadas con yema de huevo tratada con PLD suponen una mejora en relación con la yema de huevo sin tratar. Además, el empleo de PLD también conduce a un aumento de la viscosidad de yema de huevo [116, 117].

El proceso de obtención de productos alimenticios con mejores propiedades reológicas mediante adición de yema de huevo tratada con PLD también ha sido patentado [118]. Las propiedades de la yema de huevo se modifican para ser posteriormente añadidas a otros productos que requieren propiedades emulsionantes y de gelificación. Estos productos incluyen alimentos cárnicos procesados, tartas, bizcochos, galletas, helados, mayonesas, aderezos, láminas de huevo, o crepes.

Por otro lado, la PLD ha sido también añadida a los ingredientes de masa del pan antes del amasado. Esta enzima modifica los PLs de la masa junto a una PLA2, consiguiendo ambas enzimas mejorar calidad del producto terminado [119].

5.5.4 Transfosfatidilación

La PLD es una enzima hidrolítica que cataliza la ruptura del alcohol de la cabeza polar de los PLs, liberando ácido fosfatídico (PA). Sin embargo en determinadas condiciones es posible revertir su actividad hacia procesos de síntesis.

Para la mayoría de enzimas hidrolíticas, la síntesis de enlaces éster en presencia de agua está muy desfavorecida, ya que el equilibrio está completamente desplazado hacia la formación de los productos de hidrólisis. Pero la PLD es una enzima única en este sentido, ya que es capaz de transferir un alcohol al fosfolípido en presencia de agua [31]. El resultado de la reacción catalizada de transfosfatidilación por PLD es la formación de un nuevo fosfolípido modificado en su cabeza polar. Esto suele ir acompañado de cantidades variables del producto de la hidrólisis, es decir, de ácido fosfatídico. La PLD presumiblemente forma un complejo fosforilo-enzima que puede interactuar con dos moléculas; el agua y el alcohol, compitiendo ambas por unirse a la estructura del fosfolípido. La naturaleza del sitio de unión B2 marcado en la Figura 10 probablemente ejercerá la mayor influencia en la selectividad, es decir, en la relación entre el enlace formado y el producto de hidrólisis [31].

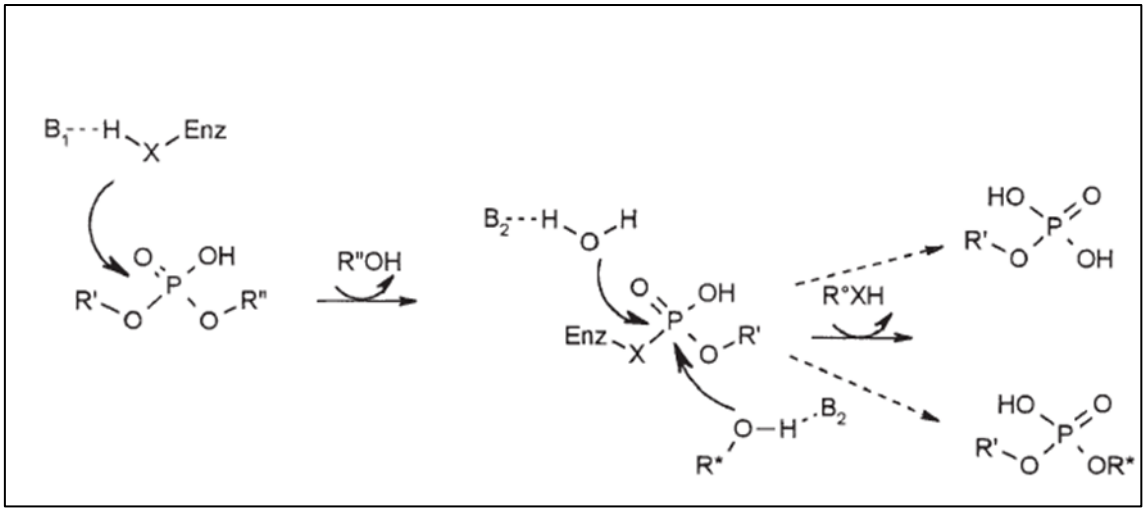


Figura 10. Complejo fosfolípido-fosfolipasa D. Adaptada de [31]

La PLD tiene la capacidad de actuar en medios bifásicos, emulsiones y microemulsiones, por lo tanto, la reacción catalítica puede ocurrir tanto en la fase acuosa con alcoholes solubles en agua, como en la fase orgánica cuando se trata de alcoholes insolubles en agua. Aunque, por supuesto, la selectividad de la reacción en

las dos fases es diferente. Durante la transfosfatidilación, la naturaleza de los componentes de la reacción está cambiando. Por lo que es probable que la partición del biocatalizador entre las dos fases vaya variando durante el progreso de la reacción. La naturaleza del disolvente por tanto, tendrá un papel destacado en la biotransformación debido a la posibilidad de formar una amplia gama de diferentes estados de agregación. El comportamiento de la PLD esta aparentemente menos influenciada por fenómenos interfaciales que otras fosfolipasas, pero también, tiende a ser más activa en presencia de interfaces [31].

Es ampliamente conocido que la PLD en la reacción de hidrólisis es calcio dependiente, aunque en algunos casos específicos, la transfosfatidilación parece no ser favorecida por la presencia de los iones de calcio. Comúnmente se reconoce que la influencia del calcio en la reacción de transesterificación es mucho menos pronunciada que en la hidrólisis con los mismos sustratos, sin embargo otros fenómenos relacionados con la transfosfatidilación sí podrían estar relacionados con la presencia de iones de calcio [31].

5.5.5 Medios de reacción en transfosfatidilación

Generalmente, las reacciones de transfosfatidilación catalizadas por PLD se llevan a cabo en sistemas bifásicos. Estos sistemas están formados por una fase acuosa y un disolvente orgánico inmiscible en agua, la fase acuosa contiene los nucleófilos solubles en agua y la enzima. Y el disolvente orgánico contiene el fosfolípido, este disolvente suele ser éter dietílico, cloroformo o acetato de etilo [120].

La elección adecuada del medio de reacción puede potenciar los beneficios de la biocatálisis en cuanto a su selectividad y a la minimización de la formación de subproducto no deseado. Por lo tanto, la elección del disolvente es crucial, ya que, las propiedades fisicoquímicas del disolvente orgánico, junto con la estructura del alcohol aceptor se muestran de manera decisiva para influir en el equilibrio entre la transfosfatidilación y la hidrólisis [64]. Esto se debe al importante papel que juega el disolvente en las velocidades de reacción, los procesos de difusión, la

desnaturalización de la enzima en el medio y el reparto de sustratos y productos entre las fases.

La aplicación industrial de la reacción de transfosfatidilación para la síntesis de nuevos PLs está generalmente limitada por su baja productividad, ya que es necesario un medio donde un reactivo polar (nucleófilo) y un fosfolípido sean capaces de reaccionar en presencia del biocatalizador. Por otra parte, para que un proceso biotecnológico pueda ser económicamente viable, deben lograrse ciertos requisitos del medio de reacción [121]. Dos de estos requisitos son de particular importancia: 1) La concentración de producto, que para ser comparable a procesos químicos clásicos debe ser al menos de 50 a 100 g / L. Dado que, en la naturaleza, las enzimas trabajan en concentraciones milimolares de sustrato, las enzimas siempre estarán operando lejos de sus condiciones naturales. Pero en este aspecto, el desarrollo y el diseño de procesos y biocatalizadores, son herramientas disponibles para lograr tales concentraciones.

2) El otro requisito relevante es el coste de la enzima y se expresa como el cociente; gramo de producto/gramo o actividad de biocatalizador. Para los procesos comerciales, este cociente debe ser 100 para una enzima y 15 para un sistema de células (que refleja los diferentes costes de cada uno). Técnicas tales como la inmovilización se vuelven importantes para proporcionar la capacidad de reciclar el catalizador (en particular si se trata de una enzima). Aunque debido a la estructura supramolecular de sustratos de PLD y los sistemas de reacción complejos necesarios, la aplicación de PLD en una forma inmovilizada es menos común que con otras enzimas. Aun así, se puede encontrar una serie de ejemplos de éxito para el uso de PLD inmovilizadas en la literatura científica [122]

Con el fin de alcanzar la productividad necesaria, la eliminación del producto de reacción es una estrategia plausible. Muchas reacciones biocatalíticas, las cuales están convirtiendo sustratos a una concentración elevada, están limitadas por la acumulación de producto en el medio de reacción, ya que éste puede degradar o inhibir el biocatalizador. Una posibilidad, es el empleo de sistemas de separación *in situ* del producto (ISPR), donde el producto, según se está produciendo, es simultáneamente separado del medio de reacción durante el proceso. Estos métodos ISPR pueden aumentar la productividad o el rendimiento de una reacción biocatalítica

por: la superación de los efectos inhibitorios o tóxicos [123]; cambio de equilibrio en reacciones desfavorables [124]; reducción al mínimo de pérdidas de producto debido a su degradación [125]; y reducción del número total de pasos de procesamiento posteriores [126]. Una segunda estrategia para mejorar la productividad, es el control en la incorporación de sustrato. La utilización de una fase auxiliar (resina sólida o disolvente orgánico), se puede utilizar para la incorporación gradual de sustrato. En muchas situaciones, en procesos utilizados en alimentación, el sustrato es inhibidor, nocivo, o insoluble a la concentración requerida, por lo que una fase auxiliar puede invertir este impedimento. Aunque el sistema bifásico y las estrategias de control de producto o sustrato puede generar un considerable aumento de la productividad, se debe tener en cuenta que la complejidad añadida debe ser compensada por los beneficios aportados [127].

Diferentes estrategias dirigidas a aumentar la productividad de la reacción han sido descritas en las últimas décadas. En cuanto al uso de resinas como fase auxiliar, ha sido diseñada una transfosfatidilación de PLD a partir de *Streptomyces sp.* utilizando un sistema de reacción con disolventes anhidros y una resina de intercambio de cationes que atrapa selectivamente colina pero no PC [128]. Además, estos autores aplican una técnica de incorporación de sal para aumentar la actividad catalítica de PLD, que fue utilizada también con éxito en la transfosfatidilación de PC por *Streptomyces antibioticus* [129].

Por otro lado, existen metodologías para eliminar inhibidores mediante el uso de otras enzimas. Éste es el caso de la eliminación de la colina libre formada mediante la acción de colina oxidasa [130].

Otra estrategia, es el sistema de reacción sólido-sólido que consigue altos rendimientos debido a que la mayor parte del sustrato se encuentra en suspensión y el producto de la reacción precipita según se va formando. Los productos insolubles que precipitan en el medio cambian el equilibrio de la reacción, lo que permite aumentar la productividad volumétrica del sistema de reacción. Se ha descrito que en estas reacciones los productos se acumulan en las fases líquidas y sólidas de la mezcla de reacción. Por tanto, pueden ser distinguidos dos diferentes tipos de rendimientos: el rendimiento del producto precipitado y el rendimiento del producto total en fase sólida y liquida. Estos rendimientos dependen del disolvente en el que se lleva a cabo

la reacción y generalmente los disolventes hidrofílicos con baja log P dan los mejores resultados en péptidos, y glucolípidos [131]. Esto se debe a la correlación inversa entre el rendimiento del producto y la solubilidad del producto[132]. Otra ventaja en esta estrategia de síntesis, es la mayor facilidad para eliminar el sustrato excedente o aislar productos de la reacción. Los sistemas de reacción sólido-sólido han sido descritos en síntesis de diferentes compuestos, tales como, glucósidos, glucamidas, ésteres, péptidos y antibióticos, aunque no han sido descritas en la síntesis de PLs [132, 133].

Un problema añadido, es la particular preocupación existente por el uso de disolventes orgánicos, que requieren instalaciones a prueba de incendios, además de ser tóxicos y contaminantes. Debido a la influencia ejercida por la "química verde" y "sostenibilidad", el empleo de disolventes orgánicos tóxicos tiende a ser evitado.

Por otro lado, este tipo de sistemas bifásicos, que en muchos casos, contiene una gran cantidad de agua, provoca un serio inconveniente, ya que da lugar a la reacción de hidrólisis no deseada. Esto, produce dos problemas principales: 1) la hidrólisis consume parte del sustrato y 2) se produce la acumulación de cantidades considerables de ácido fosfatídico (PA). En este sentido, una opción ideal para llevar a cabo la síntesis de nuevos PLs catalizada por PLD, sería un sistema de reacción no acuoso para evitar la hidrólisis y evitando además disolventes tóxicos. En este sentido, el uso de líquidos iónicos como medio de reacción ha sido ya descrito [134, 135]. Otros disolventes "verdes" podrían, en algunos casos, proporcionar también una alternativa adecuada [136]. Por otro lado, medios de reacción acuosos sin disolventes orgánicos también han sido estudiados hace años [113]. En este trabajo se observó que los sustratos simplemente dispersados en un tampón acuoso, junto con la PLD, no proporcionan un medio adecuado para producir transfosfatidilación. Pero la reacción si es efectiva si se utiliza un sistema en suspensión. En este caso, el medio de reacción está compuesto por lecitina adsorbida en sílice, o acompañada de sulfato de calcio en una solución que contiene serina y PLD. Esta reacción obtuvo altas conversiones de PS, sin embargo, estas metodologías sólo son solo capaces de producir PS a bajas concentraciones.

Un caso especial, es el de los líquidos iónicos utilizados como alternativa a disolventes orgánicos en biocatálisis. Los líquidos iónicos son sales orgánicas compuestas simplemente por iones, que son líquidos a temperatura ambiente. Estos líquidos

iónicos se han utilizado principalmente con lipasas [137], aunque también con otras enzimas hidrolíticas tales como, proteasas y glicosidasas. Las principales observaciones al utilizar estos medios de reacción, son el aumento de la estabilidad de la enzima y de la actividad catalítica [138]. Sin embargo, el uso de líquidos iónicos en la modificación biocatalítica de PLs ha sido poco investigada. El principal objetivo de la aplicación de líquidos iónicos en transfosforilaciones con fosfolipasa D, además de no ser contaminantes, es la modificación de la selectividad de la enzima consiguiendo así la supresión de reacciones de hidrólisis no deseadas. La disminución o ausencia de agua implica la reducción de la reacción de hidrólisis, haciendo que la purificación del producto sea mucho más fácil y más barata al limitar el uso de disolventes orgánicos. Aunque, el efecto de estos nuevos disolventes sobre la estructura y actividad de la fosfolipasa, y sobre la agregación de los PLs requiere más estudios. En particular, en un reciente trabajo se han estudiado líquidos iónicos comerciales como co-solventes para mejorar la síntesis de PS por transfosfatidilación. Los resultados indican que el líquido iónico conduce sorprendentemente a la supresión prácticamente total de la hidrolisis, proporcionando así una gran mejora en la purificación del producto final. [138].

5.6 FOSFOLÍPIDOS ESTRUCTURADOS MEDIANTE EL USO DE PLD

El desarrollo actual de la biocatálisis ha logrado producir fosfolípidos que proporcionan un beneficio para la salud más allá de su función nutricional, tales como, la prevención de ciertas enfermedades crónicas y degenerativas. Varias modificaciones pueden ser diseñadas utilizando fosfolipasas para formar compuestos bioactivos o mejorar su aplicabilidad tecnológica. La transfosfatidilación mediante el uso de PLD ha sido descrita para incorporar interesantes moléculas como vitaminas y antioxidantes a la cabeza polar del fosfolípido. Además, la transfosfatidilación también ha sido descrita para producir otros PLs de difícil obtención que se encuentran en la naturaleza, del mismo modo, la hidrolisis de PLs con PLD también ha sido estudiada para producir compuestos de interés como el ácido fosfatídico.

La producción de PLs difíciles de obtener de fuentes naturales a partir de PC ha sido muy estudiada. De hecho, la mayor parte de los PL podrían ser sintetizados

enzimáticamente a partir de PC [108]. Este es el caso de la PS, que ha mostrado propiedades bioactivas interesantes. Es bien conocido que la PS es un activador de la proteína quinasa C y que regula las actividades de diversas enzimas, tales como la Na^+/K^+-ATPasa [139]. Además, en ensayos clínicos llevados a cabo en EE.UU. y Europa, se ha indicado que la PS suplementada en la dieta juega un importante papel en el apoyo de las funciones mentales y en el envejecimiento cerebral [8]. Sin embargo, la disponibilidad de PS a partir de fuentes naturales, como el cerebro de animales, es muy limitada[108]. Por lo tanto, se han desarrollado alternativas como la transformación de PC a PS usando PLD de *Streptomyces sp* [130, 140].

La cardiolipina es otro PL interesante producido a partir de otro fosfolípido más fácil de obtener fosfatidilglicerol (PG). Su estructura de difosfatidilglicerol le confiere propiedades bioactivas muy atractivas. Estudios de la cardiolipina han proporcionado evidencia de la importancia que tiene en la estructura de diversos complejos de proteínas mitocondriales[141, 142]. Aunque no hay evidencia directa que apoye una relación causal entre el contenido alterado de cardiolipina y un estado de enfermedad particular, diversos experimentos han indicado una correlación entre el contenido de cardiolipina y una función celular aberrante [141]. A pesar de la escasa información, es interesante señalar que, existe un interés potencial en la suplementación con cardiolipina. La PLD se ha utilizado para sintetizar cardiolipina en dos pasos a partir de dos moléculas de PG, en primer lugar, se hidroliza una molécula de PG en PA y glicerol y seguidamente se incorpora el PA en la cabeza polar de la segunda molécula de PG mediante transfosfatidilación [143].

Las vitaminas son bien conocidas por sus actividades biológicas relevantes y por su potente actividad antioxidante. Sin embargo, su inclusión en alimentos se ve limitada si tiene una naturaleza lipófila o hidrófila diferente a la naturaleza de la matriz alimentaria a la que se desea añadir. Como ejemplo, el alfa tocoferol y sus homólogos son ampliamente utilizados como aditivos antioxidantes en diversos alimentos, incluyendo aceites comestibles y emulsiones de aceite, sin embargo, no siempre proporcionan una protección eficaz contra la rancidez oxidativa en alimentos dependiendo de su naturaleza. Ha sido descrita la síntesis de un derivado de PC que contiene vitamina E utilizando PLD, consiguiendo que el nuevo producto sea soluble en agua [144, 145], el producto sintetizado aporta mayor estabilidad oxidativa en

manteca de cerdo que el alfa tocoferol por sí solo. Además, este derivado de vitamina E puede ser incorporado en una amplia gama de productos con alto contenido en agua.

Por otro lado, el ácido ascórbico es uno de los antioxidantes solubles en agua más utilizados en la industria alimentaria. En este sentido el 6-fosfatidil-L-ácido ascórbico ha sido sintetizado con una PLD, consiguiendo un derivado más apolar de esta vitamina [146]. Como resultado se ha observado que este derivado muestra un mayor efecto antioxidante que el propio ácido ascórbico. El derivado del ascórbico posicionado en la bicapa lipídica de las membranas, puede mostrar una excelente actividad antioxidante contra la peroxidación producida en la superficie de la membrana.

Otras vitaminas solubles en agua también pueden ser modificadas, formando un derivado fosfolípido-vitamina más lipófilo. La PLD ha sido descrita en la síntesis de derivados de tiamina, ácido pantoténico y riboflavina mediante tranfosforilaciones, a partir de PC [147].

Un caso aparte son los compuestos fenólicos. Diversos estudios han demostrado que los efectos antioxidantes de compuestos fenólicos en matrices oleosas pueden mejorarse[148]. La modificación de compuestos fenólicos para la producción de nutraceuticos o ingredientes funcionales más lipofilos puede ser llevada a cabo mediante transfosfatidilación con PLD. Los fenilalcanoles, tales como el tirosol y el hidroxitirosol (HT) son antioxidantes fenólicos naturales ampliamente conocidos y con interesantes propiedades bioactivas. En cuanto a su modificación, se han estudiado derivados de HT con un mejor balance lipófilo/hidrófilo en su potencial uso como antioxidante en alimentos y en su mejora de biodisponibilidad [90]. Por lo tanto, la incorporación de tirosol e HT a PC ha sido estudiada, en este caso la transfosfatidilación se llevó a cabo mediante una PLD de *Streptomyces sp* [149]. Por otro lado, alcoholes terpénicos también han sido utilizados para obtener derivados de fosfolípidos. Estos terpenos son compuestos fenólicos con un isoprenoide presentes en aceites esenciales de plantas. El perillil alcohol es un monoterpeno cíclico de origen natural que se ha relacionado con efectos apoptóticos sobre líneas celulares de cáncer de próstata y actividad inhibidora de la angiogénesis [150]. Tras la incorporación a la estructura de PC de este y otros alcoholes monoterpénicos con PLD (fosfatidil-

perílilalcohol, mirtenol y nerol), estos compuestos mostraron un marcado efecto antiproliferativo en estudios celulares de próstata humana PC-3 y leucemia humana HL-60 [151].

En cuanto a los compuestos bioactivos producidos por la hidrolisis de PLs, destaca el ácido fosfatídico (PA), implicado en diversos procesos celulares como; transducción de señales, transporte a través de la membrana, secreción, o reorganización del citoesqueleto. Los efectos del PA se han relacionado con la supervivencia, proliferación, y la reproducción de las células u organismos [152]. Además, una mezcla de lípidos con PA ha sido patentada como tratamiento de cáncer y síndromes de abstinencia [153]. En cuanto a la obtención de PA mediante transforforilación, la reacción de hidrólisis de PC procedente de yema de huevo ha sido descrita, utilizando PLD de col y de *Streptomyces chromofuscus* [154]. El ácido lisofosfatídico (LPA) [155] [155], similar al ácido fosfatídico pero con un sólo ácido graso, posee varias funciones metabólicas. Actúa como un intermediario importante en los procesos de transducción de señales transmembrana, estimula la proliferación celular y es un precursor del factor de activación plaquetario [156]. Recientemente, se ha demostrado que el LPA está implicado en la motilidad de células tumorales y protege células epiteliales intestinales [157]. La producción de LPA también ha sido estudiada, el LPA fue obtenido mediante dos reacciones de hidrolisis, en primer lugar el PA fue liberado mediante una PLD y a continuación, fue eliminado un ácido graso con una PLA [156].

5.7 EXTRACCIÓN Y AISLAMIENTO DE FOSFOLÍPIDOS

La producción de un fosfolípido estructurado requiere un primer paso de biocatálisis enzimática y un segundo paso de purificación. Durante la primera fase, se ensaya una reacción diseñada para sintetizar la nueva molécula, a continuación, es necesaria una fase de aislamiento del compuesto sintetizado que permita obtenerlo con el mayor grado de pureza posible.

El aislamiento y la purificación de las diferentes especies moleculares de PLs, es uno de los retos de investigación actual más importantes y de mayor dificultad en el campo de la bioquímica [158]. El avance en estas tecnologías podría alcanzar un gran desarrollo

en diferentes campos; en alimentación por la producción de nuevos ingredientes funcionales; en farmacia y medicina por el desarrollo de biomembranas, liposomas y lípidos portadores intracelulares; y en la industria química optimizando la agregación y dispersión de nanomateriales [158].

El concepto de aislamiento o extracción de PLs engloba al menos tres variantes, ya que se distinguen diferentes procesos teniendo en cuenta el producto de partida del que se va a extraer el fosfolípido y el grado de pureza obtenido. Se pueden diferenciar la extracción de PLs y lípidos neutros de una matriz compleja, el fraccionamiento de PLs procedente de una mezcla lipídica y la purificación de PLs.

El aislamiento de PLs más básico, se refiere a la extracción de la fase lipídica de una masa celular, donde el producto obtenido puede ser una mezcla de PLs y lípidos neutros. Éste es el caso del ya mencionado proceso de obtención de lecitina comercial a partir de aceites de soja en bruto, aceites de girasol, y también de yema de huevo [42]. Durante el procesamiento del aceite, los PLs presentes en el aceite crudo se eliminan con el fin de mejorar la estabilidad del aceite. Para la obtención de aceites comerciales de lecitina a partir de aceite de soja crudo, se suelen incluir operaciones como hidratación de fosfátidos, separación de lodos de lecitina y secado de lodos[10]. Por otro lado, el desgomado enzimático es una variante del desgomado clásico en la extracción de aceites. Diferentes fosfolipasas y su combinación ayudan a la eliminación de PLs difícilmente extraíbles, modificando la selectividad del proceso [159, 160]. Las gomas producidas después de cualquier proceso de desgomado contienen cantidades variables de agua, materias en suspensión y aceite. Otros métodos de aislamiento de lípidos neutros y PLs procedentes de matrices alimentarias, es la extracción con disolventes, esta extracción suele ser más utilizada en el ámbito científico, como paso previo a la elucidación de los compuestos. En el caso de los PLs de soja, estos son solubles en hidrocarburos alifáticos, hidrocarburos aromáticos e hidrocarburos halogenados, tales como éter, benceno, cloroformo y éter de petróleo y en particular, son también solubles en alcoholes alifáticos, como por ejemplo, etanol. Como otros tensioactivos, los PLs de la soja son insolubles en disolventes polares como acetato de metilo o especialmente acetona [158]. Durante muchas décadas dos principales metodologías han sido utilizadas y [161, 162] ambas se basan en la incorporación de diferentes mezclas de disolventes, para conseguir disolver la totalidad de los PLs.

Seguidamente los disolventes se evaporan a vacío, el resultado suele ser una mezcla de PLs acompañado de lípidos neutros.

En cuanto al fraccionamiento de PLs de interés procedente de una mezcla lipídica, el objetivo es enriquecer la composición de PLs o un tipo de fosfolípido, mediante la eliminación de los compuestos que carezcan de interés para la aplicación final. La obtención de mezclas ricas en PL, se ha logrado tradicionalmente a través de precipitación con acetona con posterior centrifugación y secado. Este procedimiento elimina los lípidos neutros [128] a partir de mezclas que contienen PLs, sin embargo, los NL no son eliminados por completo cuando las mezclas contienen PLs con cargas. Pueden ser aisladas fracciones de PLs debido a sus diferentes solubilidades en disolventes orgánicos. La PC exhibe una gran solubilidad en alcoholes de cadena corta C1-C4), mientras que PE y PI son poco solubles en este tipo de alcoholes. Por ejemplo ha sido descrito con buenos resultados el empleo de isopropanol para separar PC de PE y PI. El fraccionamiento por la adicción de etanol en varios ciclos, con posterior centrifugación y secado, es un proceso de fraccionamiento de PLs muy común. Esta metodología se basa en que la PC es soluble en etanol, mientras que el PI no lo es, y la PE presenta una porción soluble e insoluble equivalente en etanol [10]. Por otro lado, la extracción con alcoholes inferiores junto con sales o ácidos es también un método eficaz para fraccionar PLs, se basa en la capacidad de los PLs de reaccionar con algunas sales o ácidos y precipitar. Este método es más prometedor que la extracción con disolventes orgánicos, ya que algunos iones metálicos o ácidos pueden interaccionar con ciertos PLs de forma más selectiva que los disolventes. Otros métodos también utilizados para aislar PLs de mezclas lipídicas son la cromatografía en capa fina y la extracción en fase solida [163].

Por último, el aislamiento de PLs, también conocido como purificación, se basa en la obtención de un único fosfolípido o de una familia de PLs de interés. Para obtener productos de alta pureza, se requieren generalmente varios pasos de purificación; el fraccionamiento con disolventes y el aislamiento con cromatografía preparativa a partir de fuentes comerciales. Las diferentes características entre las especies, como PLs con cargas y PLs neutros conllevan diferencias de solubilidad en ciertos disolventes o mezclas de disolventes. Como ejemplo, la purificación de PE con disolventes se basa en el uso de hexano, anhídrido acético y acetona. Por otro lado, el aislamiento

mediante HPLC preparativo usa una columna de sílice y diferentes disolventes como fase móvil (hexano/isopropanol/agua) [164-166]. Otra variante, es el uso de disolventes seguidos de ultrafiltración, en la que solo los componentes de cierto tamaño atraviesan una membrana y pueden ser así separados del resto de la muestra. La lecitina obtenida por ultrafiltración de Archer Daniels Midland Co. tiene la propiedad de ser fácil de mezclar con otros materiales, siendo de alta calidad y pureza [158].

En los últimos años, la química verde se ha convertido en un espacio de interés en la investigación científica, que busca reducir la utilización o generación de sustancias peligrosas en el diseño, fabricación y aplicaciones de productos químicos. Tradicionalmente el aislamiento de PLs se ha logrado mediante el uso de disolventes, por lo tanto, se han buscado alternativas no contaminantes. Una de las más atractivas es la tecnología de fluidos supercríticos, el dióxido de carbono supercrítico puede ser utilizado para separar LN a partir de una mezcla de lípidos que incluyen lecitinas. La extracción de los LN se vuelve cada vez más difícil, ya que el nivel de PLs en la mezcla aumenta. Este hecho se puede solventar mediante la adición de cosolventes al dióxido de carbono o a mezclas de propano/dióxido de carbono. El cosolvente mas estudiado es el etanol, por su carácter poco contaminante y porque la adición de una pequeña cantidad de etanol aumenta sustancialmente el rendimiento de la extracción [167-169].

5.8 ANÁLISIS DE FOSFOLÍPIDOS POR HPLC

Las técnicas cromatográficas son muy variadas, pero en todas ellas, hay una fase móvil (gas, líquido o fluido supercrítico) que arrastra la muestra a través de una fase estacionaria. Esta fase estacionaria es un sólido o un líquido fijado en un sólido, de este modo, los componentes de la mezcla a separar interaccionan con la fase estacionaria de manera diferente, se van separando y eluyen a distintas velocidades. Después de que los componentes hayan pasado por la fase estacionaria, separándose llegan al detector que genera una señal proporcional a su concentración y al tipo de compuesto [170].

Generalmente, el análisis de PLs necesita dos pasos previos al propio análisis por técnicas cromatográficas, uno es la extracción previa de lípidos y otro el aislamiento de la fracción de fosfolípidos. En el ámbito científico, los PLs han sido extraídos desde hace varias décadas mediante un método basado en la extracción con cloroformo y metanol. Este método [162] extrae todo tipo de PLs en diversas matrices y ha demostrado, por ejemplo, ser el más fiable para extraer los PLs de matrices como la leche [163].

En cuanto al aislamiento de la fracción de PLs de otros tipos de lípidos han sido utilizados diferentes métodos. Los métodos más empleados en el aislamiento de la fracción de PLs previa al análisis son; la cromatografía en capa fina [171, 172], la cromatografía en columna [173] y la extracción en fase solida [174, 175], sin embargo, este paso puede ser obviado en determinadas circunstancias, ya que en ocasiones, la extracción de PLs con disolventes puede ser suficiente para ser directamente analizada mediante cromatografía liquida.

La cromatografía liquida de alta resolución (HPLC) en fase normal unida al detector evaporativo de dispersión de luz (ELSD) ha sido el método analítico más utilizado para el análisis de PLs [172, 176]. Aunque, la fase reversa también ha sido descrita en multitud de ocasiones para la identificación de diferentes PLs [177].

La cromatografía en fase normal utiliza una fase estacionaria polar y una fase móvil apolar. Esta técnica se caracteriza por separar los compuestos en base a los grupos funcionales polares presentes en la molécula, no teniendo apenas relevancia en la separación, las cadenas alquílicas o grupos hidrófobos. Este hecho posibilita la separación de clases de lípidos independientemente de la longitud y grado de insaturación de la cadena hidrocarbonada. No obstante, la estructura total de la molécula afecta a la separación.

Existen diferentes detectores que se pueden acoplar a la salida de la columna de un equipo de HPLC con el fin de detectar los solutos a medida que eluyen por la fase móvil.

Con el detector ELSD, la fase móvil se evapora completamente por acción de una corriente de aire o nitrógeno en una cámara climatizada (Figura 11), mientras que los PLs y demás lípidos no son volatilizados sino simplemente nebulizados. Tras su nebulización se les hace incidir un haz de luz, y la dispersión que producen de dicha

luz, es proporcional a la cantidad de analito presente, permitiendo así su cuantificación. Las áreas de los picos son utilizadas para cuantificar los solutos, usando curvas de calibración mediante soluciones estándar de concentración conocida. Una de las principales ventajas del ELSD frente a otros detectores como los de fluorescencia, UV-visible o fotodiodos es su enorme versatilidad, ya que es un detector de masa y permite la detección y la cuantificación de metabolitos, tengan o no grupos cromóforos en su estructura. Este detector puede ser considerado universal en su aplicabilidad excepto para compuestos volátiles, ya que es capaz de responder a cualquier soluto que no se evapore antes de pasar por el haz de luz [178]. Este instrumento ofrece excelentes resultados en condiciones de elución de gradiente, es simple y robusto en su uso. La sensibilidad es comparable a la de un detector de índice de refracción , pero el detector de dispersión de luz por evaporación no se ve afectada por los cambios en la fase móvil o pequeñas variaciones en la temperatura ambiente o en la velocidad de flujo de la fase móvil [179].

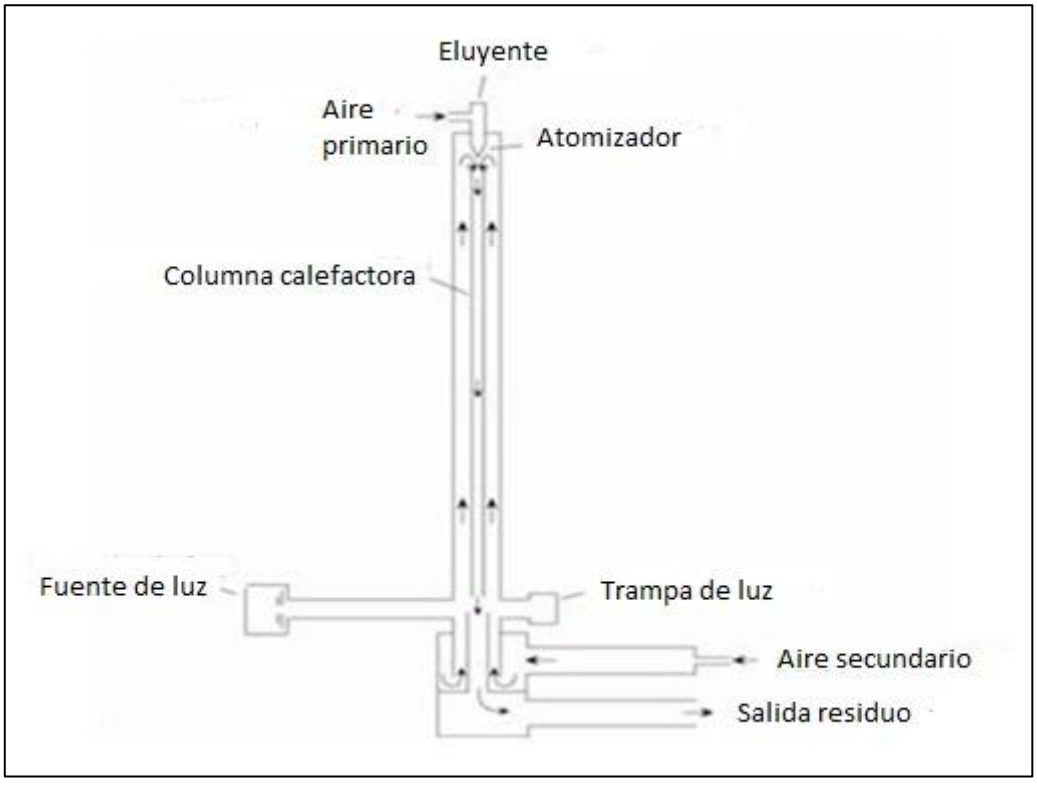


Figura 11. Diagrama del detector evaporativo de dispersión de luz (ELSD)

6 PLAN DE TRABAJO

Según los antecedentes expuestos y los objetivos planteados en esta tesis, se siguió el plan de trabajo que se describe a continuación mostrado de forma esquemática en la figura 12.

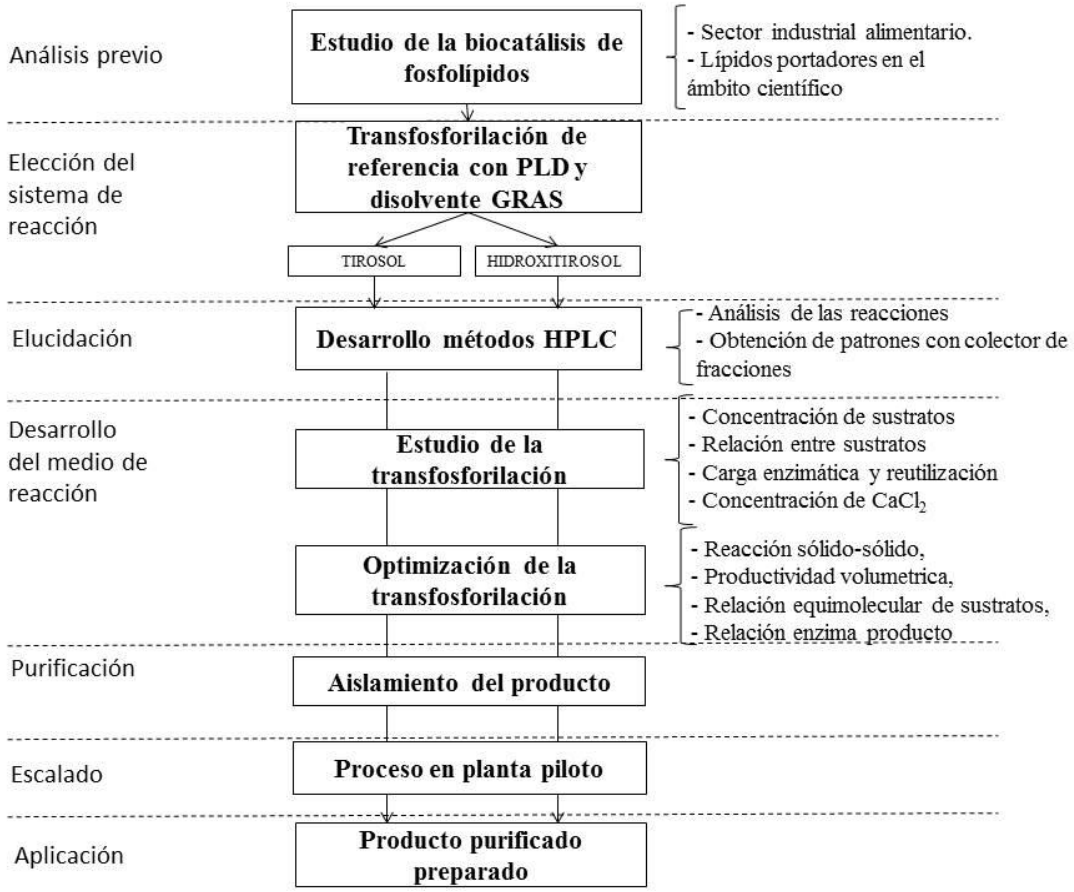


Figura 12. Esquema general del plan de trabajo llevado a cabo en esta memoria.

RESULTADOS Y DISCUSIÓN

7 RESULTADOS Y DISCUSIÓN

7.1 ARTÍCULOS INTEGRADOS EN LA MEMORIA

1. Carlos Torres, Diana Martín, Guzmán Torrelo, Víctor Casado, Oscar Fernández, Daniel Tenllado, Luis Vázquez, Inés Morán-Valero, Guillermo Reglero. (2011) "Lipids as delivery systems to improve the biological activity of bioactive ingredients." **Current Nutrition and Food Science 7(3): 160-169**

2. Víctor Casado, Diana Martín, Carlos Torres, Guillermo Reglero. (2012). "Phospholipases in Food Industry: A Review". Lipases and Phospholipases. **Methods in Molecular Biology series, G. Sandoval, Humana Press. 861: 495-523.**

3. Víctor Casado, Guillermo Reglero, Carlos Torres. (2013). "Production and Scale-up of phosphatidyl-tyrosol catalyzed by a food grade phospholipase D." **Food and Bioproducts Processing 91(4): 599-608.**

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Lipids as Delivery Systems to Improve the Biological Activity of Bioactive Ingredients

Carlos F. Torres[1]*, Diana Martín[1], Guzmán Torrelo[1], Víctor Casado[1], Oscar Fernández[1], Daniel Tenllado[2], Luis Vázquez[1], María I. Morán-Valero[1], Guillermo Reglero[1]

[1]*Departamento de Producción y Caracterización de Nuevos Alimentos. Instituto de Investigación en Ciencias de la Alimentación (CIAL) (CSIC–UAM), 28049 Madrid, Spain*

[2]*Critical Enzymes S.L. Fundación Parque Científico de Madrid. c/ Faraday, 7 Campus de la Universidad Autónoma de Madrid. 28049 Madrid, Spain*

Abstract: Delivery systems in the form of lipids have been mainly developed for drugs in the pharmaceutical field, but lipids as delivery systems for bioactive ingredients can be extended to the functional food and nutraceutical fields. Lipid delivery systems may have considerable benefit by enhancing the transport of important bioactive molecules to different tissues, as well as improving the oral bioavailability of poorly water-soluble compounds. On the other hand, formation of lipid delivery systems leads to the lipophilization of compounds, which enhances the lipophile/hydrophile balance of molecules. Furthermore, mutual activity when the carrier used is another biologically active compound is of interest for lipid delivery systems.

This review focuses on lipids as delivery systems of carrier-linked bioactive ingredients, namely triacylglycerols, phospholipids, fatty acids and fatty alcohols, sterols and alkylglycerols, as well as their inherent biological activity. The influence on the chemical stability, bioactivity, bioavailability or organ selectivity is addressed. In addition, the impact of lipophilization on other specific effects such as antioxidant will be described. Lipid delivery systems as carriers of bioactive fatty acids, phenolic compounds and vitamins will be the main examples illustrated.

Keywords: lipid delivery systems, functional ingredients, bioactive fatty acids, phenolic compounds, vitamins.

INTRODUCTION

Bioactive molecules claiming a potential use as functional ingredients or nutraceuticals are continuously appearing in the scientific literature, including compounds of diverse origin and nature such as phenolic acids, sterols, fatty acids or vitamins [1-5]. However, many of them have limited solubility, which makes difficult their manipulation for inclusion in foods, and which may limit its functionality, since the luminal solubilization and dissolution in the gastrointestinal tract are necessary for their bioavailability [6-10]. On the other hand, diverse functional ingredients are remarked due to their antioxidant properties in biological systems, but also acting as protective agents of oxidation in foods matrices. This offers an attractive advantage for their technological use as antioxidants in foods, giving an added-value to the food due to the own bioactive compound [1, 2, 7, and 11]. However, the limited solubility of diverse bioactive ingredients also affects such technological application for food manufacturers.

Oral delivery systems, natural or engineered, for the efficient release of an active compound can provide a means of optimizing the application of bioactive molecules. This strategy is extensively and intensively developing for bioactive molecules or drugs in the pharmaceutical field [10, 12, 13]. Thus, diverse strategies have been developed for drug delivery systems, such as the use of surfactants, lipids, lipid emulsions, permeation enhancers, micronization, salt formulation, nanoparticles or solid dispersions [10]. In the specific case of drug delivery systems as lipids, these include triacylglycerols (TG), diacylglycerols (DG), phospholipids (PL), fatty acids, sterols and other synthetic derivatives [14]. Within this last term of synthetic derivatives of lipids, the group of structured lipids (SL) can be included. SL are acylglycerols, glycerophospholipids or other lipid molecules that have been modified from their natural biosynthetic state by chemical and/or enzymatic methods [15]. Such modifications include the incorporation of new fatty acids or other molecules, as well as the restructuration of the lipid to change the positions of fatty acids in order to yield novel lipids.

Some examples of modified lipids for drug delivery systems can be found in the pharmacologic literature, such as GABA neurotransmitter linked to glycerides in order to promote its diffusion through the blood brain barrier [16] or L-Dopa linked to DG [17] to produce a more favorable L-Dopa/dopamine plasma and brain ratio. Pro-drugs of nicotinic acid were also prepared linked to DG esters [18], leading to a significant decrease of the free FA plasma levels in rats, without the dramatic increase in nicotinic acid plasma

*Address correspondence to this author at the Departamento de Producción y Caracterización de Nuevos Alimentos. Instituto de Investigación en Ciencias de la Alimentación (CIAL) (CSIC–UAM), 28049 Madrid, Spain; Tel: ???????????; Fax: ????????????; E-mail: ?????????????????.

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levels observed after the oral administration of an equimolecular dose of nicotinic acid. Marriot *et al.* [19] patented a product with the same therapeutic effects as valproic acid, but without causing gastric irritation, thanks to the esterification of glycerides with valproic acid.

In the food technology field, the traditional production of SL has been mainly approached to make the lipids more suitable for their own end use; namely, for food technology or nutritional objectives, such as modifications of the melting or crystallization properties of oils and fats, or for application in reduced-calorie products [20]. Taking into account the advanced state of the art of the technology of SL, the extension of their use as delivery systems of bioactive molecules for food ingredients may have considerable benefit. Similarly to drugs, delivery systems for bioactive ingredients allows the lipophilization of poor soluble molecules for application in food matrices, and the lipid delivery system may protect essential nutrients and other bioactive food components during food digestion. Thus, it may prevent the loss of the component, or adverse interactions with inhibitors of absorption. In addition, lipid delivery systems could transport important functional molecules to different tissues and enhance the oral bioavailability of poor soluble compounds. On the other hand, an additional aspect is of interest for lipid delivery systems, namely the mutual activity when the carrier used is another biologically active compound. Thus, synergistic action, additional biological activity or help to target the parent bioactive molecule to a specific site or organ and cells could be attained.

This review focuses on lipids as delivery systems of carrier-linked bioactive ingredients, namely TG, PL, fatty acids and fatty alcohols, sterols and alkylglycerols, as well as their inherent biological activity. Modifications of these structures in which specific molecules are incorporated, and the influence on the chemical stability, bioactivity, bioavailability or organ selectivity, targeting these bioactive molecules to specific areas of other tissues preventing or reducing undesirable effects, are addressed. In addition, the impact of lipophilization on other specific properties such as antioxidant effect will be described. Lipid delivery systems as carriers of bioactive fatty acids, phenolic compounds and vitamins will be the main examples illustrated.

TRIACYLGLYCEROLS AS LIPID DELIVERY SYSTEMS OF BIOACTIVE INGREDIENTS

Triacylglycerols as Lipid Delivery Systems of Bioactive Fatty Acids

TG is the traditional lipid form used to produce SL [15]. The current development of the technology of structuration of lipids allows the production of synthetic TG by the exchanging or combination of either short-, medium- or long-chain fatty acids (SCFAs, MCFAs and LCFAS, respectively) of bioactive interest. The most representative and known example are synthetic TG consisting of SCFAs or MCFAs at sn-1,3 position together with LCFAs (mainly long-chain PUFAs) or desired bioactive fatty acids at sn-2 location. This fat would integrate rapidly available energy due to the SCFAs or MCFAs, as well as essential or desired fatty acids at the same molecule. This is because fatty acids located at sn-2 position are preferentially absorbed for entering the lymphatic system, whereas SCFAs and MCFAs located at sn-1 and sn-3 positions are absorbed and transported to the liver for providing an important source of energy without being deposited in adipose tissue [20]. Therefore, it is easy to think that linking functional or bioactive fatty acids at sn-2 position of TGs is of interest on the development of lipid delivery systems of bioactive fatty acids in the form of TGs for oral administration. As examples, Maurelli *et al.* [21] synthesized TG by enzymatic reaction between sn-1,3-DG and conjugated linoleic acid isomers (CLA) at sn-2 position. Muñio *et al.* [22] produced TG with structure caprylic–PUFA–caprylic. Similarly, Haraldsson *et al.* [23] obtained a SL with DHA and EPA both in sn-2 position using a chemo-enzymatic approach.

These examples illustrate some of the possibilities of TG as delivery systems of bioactive fatty acids. The structuration of TG has been intensively studied since decades ago. For more complete information regarding studies of structured TG containing bioactive fatty acids, excellent research works and reviews found in the scientific literature are recommended [15, 20, 24, and 25]. The potential of structured TG as lipid delivery systems of other bioactive molecules different to fatty acids, such as phenolic acids or vitamins, will be reviewed in the following paragraphs, since it is considered newer and interesting for the field of delivery systems of functional ingredients.

Triacylglycerols as Lipid Delivery Systems of other Bioactive Compounds

Phenolic Compounds

Phenolic acids are natural compounds that occur ubiquitously in fruits, vegetables, spices and aromatic herbs. This heterogeneous group includes simple phenols and polyphenols as well as their derivatives. In addition to their suggested properties in biological systems as antimicrobial, anticarcinogenic and antimutagenic compounds, most phenolic acids seem to be potent antioxidants [1, 2]. Thus, the potential usage of phenolic compounds in processed foods as natural antioxidants is of current interest [1, 2]. However, the poor solubility of phenolic compounds has been reported as a major drawback that limits its application and bioavailability [26, 27]. Lipophilization of phenolic acids, which involves the esterification of the phenolic acid with a lipophilic moiety, leads to new molecules with modified lipophilic/hydrophilic balance, enhancing its utilization as antioxidant compounds and improving its bioavailability [6].

Some attempts of lipophilization of phenolic acids have been performed by complexation with liposomes, nanoparticles, niosomes or structuration with fatty acids [28]. In the specific case of structured TG, Reddy *et al.* [29] described the chemo-enzymatic synthesis of structured TG bearing ferulic acid at sn-1/3 position. Ferulic acid is a common phenolic acid which is of current interest due to both evidenced antioxidant and antiinflamatory properties in general, and that has been pointed out as potential therapeutic agent in the therapy of free radical-related syndromes such as neurodegenerative disorders, cancer, cardiovacular and skin diseases [28]. Reddy *et al.* [29] showed an improvement in the antioxidant activity of the

backbone and the vehiculated compound at sn-1, sn-2 or at polar head group, with the likely subsequent impact on the final functional activity.

Phospholipids as Lipid Delivery Systems of Bioactive Fatty Acids

Modifications of PLs by incorporation of fatty acids at sn-1 or sn-2 locations can be carried out by alcoholysis, acidolysis or esterification by diverse enzymatic methods [49]. In addition, the polar head group of PLs can be also modified or replaced with functional or bioactive alcohols by the transphosphatidylation reaction catalyzed by phospholipases D. Thus, diverse enzymatic methods to incorporate specific fatty acids into PLs using lipases and phospholipases have been reported, in order to incorporate both SCFA, MCFA and LCFA, as well as long-chain PUFA or other particular fatty acids such as CLA [50-52].

In the specific case of n-3 fatty acids and n-3 PUFA, different studies of PLs esterification with these fatty acids have shown the interest on this issue [51, 53]. This is because the PUFA-containing PL seems to be related to biological properties. Thus, PUFA-containing PC or lyso-PC in cell membrane has shown a decrease of plasma lipids or inhibition of tumor cells [54, 55]. On the other hand, *sn*-2 PUFA-containing PC seems to be a good source of entry of PUFA into the brain [56]. Huggins *et al.* [58] suggested that PC containing PUFA at *sn*-2 position caused changes in recombinant HDL structure affecting apolipoprotein A-I stability and conformation, as well as influencing the fluidity and hydration of the PL environment. In this sense, Bayon *et al.* [57] esterified DHA in the form of lyso-PC and showed that it was captured by the brain of intravenously administered rats better than the lyso-PC containing other fatty acids such as 18:1, 18:2 and 20:4.

Regardless of the specific structured PLs, it is interesting to remark the different behavior of delivery systems in the form of PLs when compared to delivery systems of the same fatty acids in the form of structured TG. Tall *et al.* [59] studied different natural sources of PUFA as TG or PL and the influence on the distribution in plasma lipoproteins. The level of arachidonic acid and DHA in HDL-PL and cholesteryl esters was significantly higher in piglets fed the PL diet than in those fed the TG diet. Opposite results were found in the LDL-PL. These evidences showed that PLs seemed to be better vehicle for improving the bioavailability of some bioactive fatty acids than that observed for TG.

Phospholipids as Lipid Delivery Systems of other Bioactive Compounds

Phenolic Compounds

Phenylalkanols, such as tyrosol and hydroxytyrosol (HT), are well-known natural phenolic antioxidants. Within other phenols, HT has been pointed out as responsible for the health benefits associated with habitual consumption of virgin olive oil. It seems to protect against oxidative stress, to reduce risks connected with aging pathogenesis, and to be active against microbial attack or cancer proliferation [60-62]. The hydrophilic antioxidant HT has been shown as a potent antioxidant in fat and oily matrices [7], hence the interest on producing lipid delivery derivative of HT. As example, tyrosol and HT have been lipophilized by phosphatidylation with phospholipase D [63].

Perillyl alcohol is a naturally occurring cyclic monoterpene that has been related to apoptotic effects on prostate cancer cell lines and angiogenesis inhibitor effect [64]. Synthetic phosphatidylated monoterpenes alcohols catalyzed by phospholipase D (phosphatidyl-perillyl alcohol, -myrtenol, and -nerol) showed a marked antiproliferative effect on human prostate PC-3 and human leukemia HL-60 cells [65].

Vitamins

Alpha-tocopherol and its homologs are widely used as antioxidant additives in a variety of foods including edible oils and oil emulsions, but these lipid soluble types of vitamin E do not always provide effective protection against oxidative rancidity in all foods, especially those of more polar nature containing high water content [66]. In this respect, the synthesis of a water soluble vitamin E derived from PC by phospholipase has been attempted [67, 68], showing higher affinity to PLs membranes [67] and higher activity in improving the oxidative stability of lard than alpha-tocopherol [69].

Concerning carotenoids, it has been shown that emulsification and, consequently, uptake of carotenoids, can be enhanced by the presence of lyso-PC [70]. The water dispersibility of hydrophobic carotenoid has been also greatly enhanced by using it as the acyl part in the synthesis of a highly unsaturated lyso-PL [71]. According to these evidences, chemical synthesis and characterization of a carotenoid-glycerophosphocholine derivative has been performed [72, 73].

L-Ascorbic acid is one of the water-soluble antioxidants in the defense system against active oxygens in the interface area of membrane lipid and aqueous phase [11]. The most popular form of lipophilic derivative of ascorbic acid is ascorbyl palmitate, but forms of lipid delivery of ascorbic acid as PL have been also described. Thus, Nagao *et al.* [74] synthesized 6-phosphatidyl-L-ascorbic acid, which showed better antioxidant effect than the own L-ascorbic acid.

Other water soluble vitamins as thiamine, pyridoxine, riboflavin and pantothenic acid can be modified by their linking to PLs through enzymatic synthesis, the PL-vitamin derivatives being lipophilic forms of water soluble vitamins. As example, a phospholipase D was used to catalyze the transfer of the dipalmitoylphosphatidyl residue from 1,2-dipalmitoyl-3-*sn*-PC to thiamin, pantothenic acid, thiamin, riboflavin and their derivatives in a biphasic system [75, 76].

FATTY ESTER AND FATTY ALCOHOLS AS LIPID DELIVERY SYSTEMS OF BIOACTIVE INGREDIENTS

Phenolic Lipids

Besides TG and PL, delivery systems of phenolic compounds by esterification to fatty acids or alcohols has been also described in diverse studies. As example of the popular ferulic acid, Chigorimbo-Murefu *et al.* [77] achieved the synthesis of esters of ferulic acid with natural compounds by transesterifications of vinyl ferulate with hydroxyl-

synthesized phenolic SL with ferulic acid compared to ferulic acid alone when they were tested both in a lipid matrix and in a micellar system of linoleic acid. The improved antioxidant activity of the SL of ferulic acid was comparable to that of the control dodecyl gallate in the lipid matrix, and superior activity than the control was observed when the SL was included in the micellar system of linoleic acid.

Xin *et al.* [30] investigated the lipase-catalyzed transesterification of ethyl ferulate with triolein, to form feruloyl oleins, whereas Sun *et al.* [31] studied the enzymatic esterification of glyceryl ferulate and oleic acid for feruloylated DG synthesis. Similarly, Zheng *et al.* [32] described an enzymatic synthesis approach for the preparation of feruloylated-structured glycerides through the transesterification of ethyl ferulate and tributyrin. These authors reasoned that structuring tributyrin with ferulic acid could produce a novel bifunctional feruloylated lipid, because ferulic acid moiety may work as antioxidant, while butyric moiety has been pointed out as a bioactive SCFA with suggested anti-inflammatory and anticancer properties [33]. However, the potential application of free butyric acid as antitumor agent beyond colon is limited by the problem to reach enough plasma concentrations required to exert its antiproliferative/differentiating actions. Moreover, it is rapidly metabolized, showing a short half-life [34]. Hence there is an interest on producing delivery systems of butyrate such as the illustrated feruloylated lipid with tributyrin. Subsequent studies showing the effective bioactivity of the produced feruloylated-structured glycerides with tributyrin are not available in the scientific literature at present.

Concerning other phenolic compounds, Karboune *et al.* [35] synthesized structured phenolic lipids by lipase-catalyzed acidolysis of flaxseed oil with hydroxylated or/and methoxylated derivatives of cinnamic, phenyl acetic and benzoic acids. These authors reported that the antioxidant activity measured by the radical scavenging activity of the derived 3,4-dihydroxyphenyl acetylated lipids was lower than that of the free phenolic acid form, but close to that of the control α-tocopherol. On the contrary, in the case of derived *p*-coumaroylated lipids, the antioxidant activity was similar to that of coumaric acid.

Sabally *et al.* [36] described the production of phenolic MG and DG by enzymatic transesterification of dihydrocaffeic acid with flaxseed oil. In this case, a negative effect on the radical scavenging ability of the modified phenolics was reported. It was suggested that the attachment of the glycerol moieties to the side chain of the dihydrocaffeic acid might reduce the rotation degree of the phenyl moiety, leading to a reduction in the radical scavenging capabilities.

Vitamins

Carotenoids are known for providing structural stability and other beneficial functions to membranes. They have been suggested as potent antioxidants and seem to protect against a variety of diseases, including cancer, cardiovascular disease and eye health [37; 38]. Unfortunately, these compounds are highly labile and susceptible to oxidation. Thus, carotenoid delivery systems have been suggested as potential forms of avoiding the carotenoid degradation and allowing the more efficient inclusion in food matrixes [38].

Houte *et al.* [39] synthesized structured glycerides containing carotenoic acid. Additionally, selena fatty acids were also included in the glyceridic molecule together with carotenoids, in order to combine two bioactive molecules within the same lipid, since it seems that the replacement of a methylene group by selenium increase the inhibition of microbial growth [40]. Moreover, selenium also seems to act as antioxidant and enhancer of the immune system [40]. On the other hand, carotenoids, vitamin E and selenium show similar or complementary physiological properties and seem to protect against a variety of pathological processes. In this sense, Naalsund *et al.* [41] esterified carotenoic acid, selena fatty acid and vitamin-E with glycerol, a molecule that was named "triantioxidant glyceride".

Phospholipids as Lipid Delivery Systems of Bioactive Ingredients

PL are major constituents of cell membranes and play crucial role in the biochemistry and physiology of cells. The parent structure of the PLs is phosphatidic acid (sn-1,2-diacylglycerol-3-phophate, PA). The term lyso-PLs is used for denoting the hydrolyzed PLs at sn-2 location. Both non-hydrolyzed PLs, lyso-PLs and PA are molecules of bioactive interest and, at the same time, might be potential delivery systems of bioactive compounds, including functional fatty acids.

The consideration of the particular intestinal hydrolysis of oral PLs is crucial for taking advantage of the potential bioactivity of the linked fatty acids to PLs. In the lumen of small intestine, the dominant digestive enzyme for PL, namely phospholipase A_2, hydrolyzes fatty acids from sn-2 location, releasing free fatty acids and lyso-PL, with a fatty acid remaining at sn-1 location. Both hydrolysis products are taken up by mucosal cells and are re-secreted within chylomicrons as newly-formed PL or TG [42]. It is easy to think that linking functional fatty acids at sn-1 position of PLs is of interest in the development of lipid delivery systems of bioactive fatty acids in the form of PLs for oral administration. In addition, regardless of the specific sn-1 fatty acid, the hydrolysis product lyso-PL is of interest by itself, considered as a lipid second messengers that evoke a variety of biological responses including immune response, platelet aggregation, cell proliferation and differentiation [43, 44]. On the other hand, during the metabolism of PLs, the enzyme phopholipase D catalyzes the hydrolysis of the phosphodiester bond of PLs to generate PA and a free headgroup. PA has been implicated in various cellular processes in signal transduction, membrane trafficking, secretion, and cytoskeletal rearrangement [45]. Lysophosphatidic acid (LPA) can be also released during metabolism of PLs. LPA has been shown to act as an intermediate in transmembrane signal transduction processes, as a platelet activating factor and in the stimulation of cell proliferation, as well as in tumor cell motility [46, 47]. In addition, LPA with different fatty acids species exhibit differential biological activities as a result of differentially activation of the LPA receptors [48].

All the described forms of non-hydrolyzed PLs, lyso-PLs, PA, as well as LPA have been tested as delivery systems of bioactive molecules, with the potential of combining within the same molecule the bioactivities of both the PL

steroids catalyzed by lipases. The lipid derivative showed higher antiradical activity against the ABTS free radical test, inhibited the oxidation of LDL more efficiently, and exhibited higher antioxidant activity than Trolox and the own ferulic acid.

The interest on the phenolic compound HT has been already described in previous sections. Despite the bioavailability of HT is not a problem, it has been recently shown that synthetic ester derivatives of this phenol were transported across differentiated Caco-2 cell monolayers more efficiently than the former HT, which suggests a better intestinal absorption of the lipophilic derivatives of HT [78]. As already explained, another reason of the interest on producing lipid delivery derivates of HT is to enhance its use as antioxidant in foods. Therefore, derivatives of HT with a better lipophile/hydrophile balance are being assayed for their possible use as antioxidants in foods and, at the same time, for the increase of bioavailability.

The most common methodology to convert HT into more lipophilic analogs is the esterification of the primary alcoholic group without affecting the catechol moiety, which is known to be essential for the antioxidant and protective effects [7]. More than 40 lipophilic analogs of HT have been produced using lipases such as *Candida Antarctica* [79]. Most of these lipophilic analogs of HT showed good radical scavenging activities and/or effectively inhibited lipid oxidation. In some cases, the HT analogs were even more effective than tocopherol or BHT. Some of the lipophilic HT analogues also demonstrated to protect towards oxidative damage to human cells, and in particular towards H_2O_2-induced DNA damage [7]. In addition, the position of the alkyl chain influenced the scavenging capacity, whereas the type of fatty acid acylating the phenolic alcohol did not seem to be relevant [7].

Flavonoids are the most common group of polyphenolic compounds in the human diet and are found ubiquitously in plants. The beneficial effects of fruits, vegetables, tea, and red wine have sometimes been attributed to flavonoid compounds. However, the use of flavonoids is limited by their low stability and solubility [8]. It has been shown that the esterification of isoflavones with fatty acids at different hydroxyl groups provided the lipophilicity needed for incorporation into LDL and increased the oxidation resistance of LDL [80]. On the other hand, the specific esterification of flavonoids with unsaturated fatty acids showed evidences of lower releasing of vascular endothelial growth factor, indicating that they might possess improved anti-angiogenic and anti-tumor properties [81]. Salem *et al.* [82] have recently shown that the enzymatic acylation of flavonoid compounds with ethyl laureate and ethyl butyrate enhanced the capacity to inhibit xanthine oxidase and its antiproliferative activity, but decreased the radical scavenging activity. Other studies have shown that the introduction of an acyl group into glucosylated flavonoids significantly improved their antioxidant activity towards both LDL and serum model *in vitro* [83]. Furthermore, the acylated derivative of disaccharidic flavonoid increased its antimicrobial activity against two Gram-positive bacteria [83].

Rosmarinic acid is a polyphenolic acid popular for being related to the prevention of oxidation of LDL, inhibition of cell proliferative activity, as well as antibacterial, antiviral and antioxidative activities [84]. The production of SL of rosmarinic acid as lipid systems of this polyphenol is scarce, but the assays about their bioactivities are promising. Thus, esters from rosmarinic acid formed by chemical procedure showed a greater free radical scavenging activity than rosmarinic acid [85]. In addition, esters reduced thrombin-evoked platelet aggregation and the ability of platelets to accumulate Ca^{2+} in the intracellular stores. These observations suggested that the structural lipid analogs of rosmarinic acid might be the base of therapeutic strategies to prevent thrombotic complications associated to platelet hyperaggregability due to oxidative stress.

Similar to most phenols, caffeic acid and its derivatives are known for antioxidant properties. Lipid derivative systems have been prepared and tested as propyl and methyl esters of caffeic acid [86]. The anticancer properties of these compounds in human cervix adenocarcinoma cells were tested and it was found a significant growth-inhibition effect for some of these compounds, clearly dependent on their structural characteristics. Thus, the propyl esters showed a considerably more pronounced antitumoral effect than their octyl and methyl analogs.

Vitamins

The most popular form of lipophilic derivative of ascorbic acid is ascorbyl palmitate. Dietary ascorbyl palmitate seems to be an effective source of plasma vitamin C [87]. It has also been demonstrated that lipid derivative of ascorbic acid inhibited the promotion of ornithine decarboxylase activity which may inhibit induced biochemical parameters associated with skin-tumor promotion [88]. In addition, a lipid-soluble derivative of ascorbic acid could serve as a carrier of ascorbate into neural tissues [89]. This last study showed that the content of the soluble derivative of ascorbic acid was higher than free ascorbic acid in cerebral cortex and carotid body tissues, by nearly an order of magnitude, after ingestion of the ascorbyl palmitate. Thus, the lipophilic derivative was able to cross biological barriers and satisfied the tissue demand for ascorbate better than the hydrophilic form.

The effect of the acylation of other vitamins for improving their bioactive properties has been also studied. Vitamin A or retinoic acid is linked to the formation of visual pigments and its deficiency has been related to poor biosynthesis of proteins in the retina [90]. Retinyl palmitate, as ester derivative of retinoic acid, was suggested to be an effective way to maintain the levels of proteins in the retina [90]. On the other hand, concerning retinoic acid, an important negative effect of this vitamin is its teratogenic effect evidenced since decades ago [91]. Nevertheless, in the rat, the derivative form as retinyl palmitate seems to be a significantly less potent teratogen than the retinoic acid form [91], which illustrates another interesting application of the production of lipid derivatives of vitamins.

STEROLS AS LIPID DELIVERY SYSTEMS OF BIOACTIVE INGREDIENTS

Sterols belong to the family of triterpenes and consist of a tetracyclic cyclopenta[a]phenanthrene structure and a side chain linked to the C-17. Cholesterol, which is mainly found

in animal tissues, is the most representative compound of the sterol family. Sterols are also found in vegetable tissues, being known as phytosterols (PS) or plant sterols. PS can be classified into sterols and stanols, according to the presence or absence of a double bond at the Δ5 position [4]. PS are currently popular as hypocholesterolemic agents due to interference with intestinal absorption of cholesterol [3]. In addition, PS have been related to anti-cancer, antiatherosclerosis, anti-inflammation and antioxidation activities [3].

The first studies about the inclusion of PS in functional foods were performed by free forms of these molecules. However, the poor solubility of free sterols led to inconsistent and confusing results in clinical studies and high doses of free sterol intake were required to achieve significant cholesterol reduction [9]. This drawback was solved when it was solubilized in the provided preparations [92]. Thus, emulsification of PS with lecithin, sucrose esters, DG or with different food matrices were suggested for increasing their bioavailability into the intestinal micelles [93]. Nevertheless, PS esterification to fatty acid has been the most common solubilization method in food preparations and dietary supplements [94-96]. Besides improving the solubilization and management of PS for inclusion in foods, the esterification of PS is currently of interest due to the potential of producing lipid delivery systems for both the own PS and the own esterified compound, which might be a bioactive molecule by itself regardless the attached PS; and even more attractive, synergistic bioactivities between both molecules might result from the process of esterification.

Sterols as Lipid Delivery Systems of Bioactive Fatty Acids

PS esterified to fish-oil fatty acids (FO-PS), as the main n-3 PUFA source, has been produced and tested in diverse studies. Consumption of FO-PS favorably affected key and independent risk factors associated with cardiovascular health disease, such as elevated plasma TG levels and LDL-cholesterol [97, 98], suggesting a combined beneficial effect of PS and PUFA. Furthermore, FO-PS supplementation had a potent hypotriglyceridemic effect that may be even more pronounced than the one observed with regular FO supplementation. On the other hand, Jones *et al.* [99] compared the effects of PS esterified to sunflower oil, olive oil, or FO in mildly overweight hyperlipidemic subjects. These authors suggested that FO-PS resulted in lowered blood TG and higher fat-soluble vitamin levels in comparison to vegetable oil FA esters of PS.

The popular sterol analogs of PS, the cholesterol, has been proposed as potential pro-drug to deliver butyric acid in the form of cholesteryl butyrate solid lipid nanoparticles (chol-but SLN) [100]. The interest on pro-drugs of butyric acid has been already explained in previous sections of this manuscript, concerning the promising bioactive properties of butyrate, but the drawback of its short half-life in plasma [34]. The synthetic form Chol-but SLN showed to be a potentially effective approach for delivering butyric acid to tumor cells, and it prolonged cell exposure to the drug. Chol-but SLN inhibited cell growth at six times lower concentration than that necessary to obtain a similar effect with free sodium butyrate. In addition, Chol-but SLN also affected the proliferation pattern of both myeloid and lymphoid cells to a greater extent than the natural butyrate [101]. Despite the lack of current information, the esterification of cholesterol to butyric acid showed the potential of producing other different structures of synthetic sterols esterified with SCFA as lipid delivery systems of the esterified fatty acids. In fact, synthetic short-chain esterified PS have been already patented, but in this case, the SL was performed for enhancing the inclusion of PS in aqueous food compositions by improving their hydrodispersibility [102].

Sterols as lipid delivery systems of other bioactive compounds

Phenolic Compounds

Sterols found in sources like corn, wheat, oat, rice bran and sheanut are frequently esterified with phenolic acids such as ferulic acid, coumaric acid, caffeic acid, and cinnamic acid [103]. The phytosteryl ferulate form has shown multiple health beneficial activities. Thus, gamma-oryzanol, a phytosteryl ferulate mixture extracted from rice bran oil, has shown reduction of cholesterol levels, inhibition of platelet aggregation and antioxidant functions [103, 104].

Chigorimbo-Morefu *et al.* [77] demonstrated the feasibility of synthesis of ferulic derivatives when reported the first biocatalysed synthesis of sterol derivatives of ferulic acid. They reported that the antioxidant activities of some of the synthesized compounds were higher than that of the precursor ferulic acid. Similarly, a recent patent [105] relates to a synthetic method for the preparation of phytosteryl ferulate using ferulic acid and PS isolated from soybean oil deodorizer distillate. The obtained compound significantly lowered the elevated cholesterol levels in hamsters and interfered with the absorption of cholesterol, the effect being comparable with that of natural oryzanol.

Vitamins

Besides esters of fatty acids, PL or TG, sterols have been also tested as lipid delivery systems of ascorbic acid. A study about the effects of different PS analogs on colonic mucosal cell proliferation in hamsters reported that plant stanols esterified to ascorbic acid may possess anticarcinogenic properties in the colon by suppressing colonic mucosa cell proliferation; however, this effect was not observed with free plant sterols or stanols [106]. Similarly, a patent [107] provided novel derivatives comprising PS/phytostanols and ascorbic acid. These derivatives afforded dietary and therapeutic advantages when compared to the use of PS/phytostanols without such attachment. Solubility of the novel derivatives was greatly enhanced, both in aqueous solutions and non-aqueous media such as oils and fats. Moreover, the formation of these derivatives allowed the full potential of ascorbic acid by avoiding its decomposition. In addition, these derivatives showed a good heat stability, which is essential for further processing.

ALKYLGLYCEROLS AS LIPID DELIVERY SYSTEMS OF BIOACTIVE INGREDIENTS

Alkylglycerols, alkylglycerophospholipids and their derivatives, namely ether lipids, are membrane components and

cellular signaling molecules. These glycerides contain O-alkyl or O-alk-1-enyl groups at sn-1 position. Glyceryl ether diesters or monoesters can be also found. Alkyldiacylglycerols are especially abundant as major components of total lipids in liver oil of marine invertebrates and vertebrates (dogfish and various other species of elasmobranch fish, especially shark liver oil) [108]. In the human body, they can be found in the cells of the immune system and in human breast milk [108].

Ether lipids have been the subject of much attention last years because of their special physiological functions in humans. Within ether lipids, the most popular one, due to important biological activities, is the platelet-activating factor (PAF), a 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine, with diverse cell mediator effects in a variety of tissues and systems, including circulation, inflammation, development and reproduction [109]. The 1-O-alkyl-sn-glycerols have been also claimed to display various beneficial effects on human health and seems to amplify the production of PAF by incorporation within the PAF precursor 1-O-alkyl-2-acyl-sn-glycero-3-phosphocholine [110]. Additionally, alkylglycerols are potent antineoplastic agents which inhibit growth, show antimetastatic activity, and induce differentiation and apoptosis in cancer cells [110]. Immune stimulation properties have also been attributed to these substances [111], as well as improving of boar sperm motility and fertility [109].

Synthetic ether lipids that do not occur in nature have been prepared to find therapeutic agents [112], such as PAF-like lipids, which retain a short-chain residue at the sn-2 position other than an acetyl group [113]. This short-chain residue may contain either a ω-methyl, ω-aldehyde, ω-alcohol, or a ω-carboxyl group for PAF-like activity. In this sense, it has been shown that the shorter the sn-2 chain residue the more active the PAF-like lipid [114, 115].

Torres *et al.* [116] have recently synthesized structured alkyldiacylglycerols containing residual short-chain fatty acids at sn-2 and sn-3 locations by enzymatic transesterification of 1-O-Octadecyl glycerol and ethyl butyrate. The potential bioaccessibility of this synthetic alkylglycerol was later assayed by simulating the intestinal digestion of the molecule by pancreatic enzymes under *in vitro* conditions [117]. This study showed that the suggested bioactive structure of 1-O-alkyl-sn-glycerol retaining a short-chain residue was produced after intestinal hydrolysis as the major hydrolysis product, in the form of 2-butyroil-1-O-octadecyl glycerol. On the other hand, in the production of this structured alkylglycerol, Torres at al. [116] claimed that this molecule might have an additional interest taking into account that the short-chain residue at sn-2 location of this lipid was the bioactive fatty acid butyrate. It has been stated that efficient pro-drugs of butyrate should have a sufficiently stable bond between the carrier and butyrate residue to increase its *in vivo* half-life [118]. In the performed *in vitro* intestinal digestion study [117], it was shown that the traditional tributyrin proposed as pro-drug of butyrate was completely hydrolyzed to butyric acid, in contrast to the obtained stable esterified form of butyric acid as 2-butyroil-1-O-octadecyl glycerol, showing the potential of this synthetic molecule as carrier of butyrate.

Another recent example of ether lipids as lipid delivery systems showed the synthesis of alkyl hydroxytyrosol ethers [119]. The obtained derivatives retained the protective antioxidant capacity of HT in oil matrices. In addition, the efficient bioavailability of these HT ethers was demonstrated [120].

Other studies of synthetic alkyldiacylglycerides as lipid delivery have not been found in the scientific literature, but it sounds a promising field for delivery systems of bioactive compounds, with the additional advantage of the own bioactivity of the alkylglycerol molecule.

CONCLUSIONS

Diverse bioactive molecules claiming a potential use as functional ingredients or nutraceuticals have a limited solubility or high lability, which makes their application difficult in the food formulation, and which may limit its functionality and bioavailability. In addition, the poor solubility of bioactive ingredients showing antioxidant properties may also affect the potential technological application for food manufacturers as antioxidant compounds for food protection. The current development of the technology of SL, especially by enzymatic methods, is allowing the production of lipid delivery systems of bioactive molecules for food ingredients that, similarly to drug lipid delivery systems in the pharmacology field, allows the lipophilization of poor soluble molecules for application in food matrices. In addition, lipid delivery systems could transport important functional molecules to different tissues and enhance the oral bioavailability of poorly soluble compounds.

The reviewed examples have successfully illustrated the validity of synthetic lipid delivery systems for improving the bioactivity and incorporation of functional ingredients in foods. Thus, diverse lipid delivery systems including TG, PL, fatty acids and fatty alcohols, sterols and alkylglycerols have been tested as carrier-linked of functional compounds such as bioactive fatty acids, phenolic compounds and vitamins. In some cases, improved bioactivities were shown for the synthetic compounds. On the other hand, the mutual activity where the carrier used is another biologically active compound, such as PL, sterols or alkylglycerols, is an additional aspect of interest for the development of lipid delivery systems for functional foods applications.

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Chapter 29

Phospholipases in Food Industry: A Review

Víctor Casado, Diana Martín, Carlos Torres, and Guillermo Reglero

Abstract

Mammal, plant, and mainly microbial phospholipases are continuously being studied, experimented, and some of them are even commercially available at industrial scale for food industry. This is because the use of phospholipases in the production of specific foods leads to attractive advantages, such as yield improvement, energy saving, higher efficiency, improved properties, or better quality of the final product. Furthermore, biocatalysis approaches in the food industry are of current interest as non-pollutant and cleaner technologies. The present chapter reviews the most representative examples of the use of phospholipases in food industry, namely edible oils, dairy, and baking products, emulsifying agents, as well as the current trend to the development of novel molecular species of phospholipids with added-value characteristics.

Key words: Phospholipids, Phospholipases, Degumming, Dairy products, Bakery, Emulsifiers, Functional foods

1. Introduction

Phospholipases are fundamental enzymes that play a crucial role in living organisms in general and in the metabolism and biosynthesis of phospholipids (PL) in particular (1). Specifically, three general functions can be ascribed to the physiologic relevance of phospholipases: (1) membrane maintenance and remodeling, (2) regulation of cellular mechanisms and signal transduction and (3) digestive role (2). Therefore, due to basic functions of phospholipases, it is understandable the finding of these enzymes in most living organisms, including plants, animals, fungi, and bacteria.

Besides the crucial biological role of phospholipases, these enzymes have reached an important function in the food industry on the simple basis that (1) PL are present in most foods and (2) the modification of PL of foods leads to advantages in diverse applications. Thus, the use of phospholipases for food processes

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can lead to yield improvement, energy saving, higher efficiency, improved properties, or better quality of the final product (3, 4). Additionally, the use of phospholipases allows the enrichment, purification, or obtaining of particular species of PL for which an appropriate natural source is not available, or creating novel molecular species of PL with added-value characteristics, such as structured PL (4). On the other hand, an attractive reason for including enzymatic technologies in the food industry can be related to the present interest on non-pollutant and cleaner technologies, the inclusion of biocatalytic approaches in the food industry being accepted as a useful alternative. Thus, mammal, plant, or microbial phospholipases are continuously being studied, experimented, and some of them are even commercially available at industrial scale. Specifically, the use of those of microbial origin are the most cost-effective and environmental friendly (5).

The most representative examples of the use of phospholipases in food industry can be found in the production of edible oils, dairy, and baking products or emulsifying agents. Thus, phospholipases are incorporated in processes such as the degumming of vegetables oils during refinement for removing undesirable compounds, the manufacture of cheese for yield increasing, or the production of bread as bakery improvers for reducing the inclusion of emulsifying compounds (5).

The present chapter reviews the current trends on the main phospholipases and their application on food industry, showing both those enzymes that are commercially available and those that are still under experimental studies but with potential interesting applications in the production of traditional foodstuffs, as well as novel foods or ingredients with improved health or bioactivities characteristics.

2. Phospholipases for Food Industry

Phospholipases form a large class of enzymes with wide diversity. The simplest classification groups of the phospholipases are within two general sets: (1) acyl hdyrolases and (2) phosphodiesterases. Each set of phospholipases is represented by different groups depending on the site of action within the PL molecule, taking into account that PL have two carboxylic ester bonds and two phosphate ester bonds.

The acyl hydrolases include the phospholipase A_1 (PLA_1), phospholipase A_2 (PLA_2), phospholipase B (PLB) and lysophospholipase $A_{1/2}$ ($lysoPLA_{1/2}$). The acyl hydrolases remove and replace the acyl chain by mechanisms of hydrolysis, esterification, and transesterification. The phosphodiesterases are represented by phospholipase C (PLC) and phospholipase D (PLD) (2, 6). In addition,

each family has many subgroups or isoenzymes belonging to the same groups. Thus, hundreds of phopholipases have been purified, characterized, or cloned from different organisms (1).

It is important to point out that except PLA2 most of these phospholipases are not widely available at large quantities for industrial purposes (1). Nevertheless, their separation, characterization, and cloning, which is being performing intensively during last decades, is an important starting point for potential applications of more diverse phospholipases at industrial scale. One of the recent approaches for broadening the use of enzymes in biocatalysis is the protein engineering technology, which means the creation of new proteins by genetically modifying already existing ones, conferring desirable industrial properties, such as substrate specificity, stability, efficiency, or pH optima (6).

2.1. PLA_1

The PLA_1 (EC 3.1.1.32) is a ubiquitous enzyme found in nearly every cell where it has been tested, including metazoan and protozoan parasites and snake venoms (2). The PLA_1 specifically hydrolyzes 1-acyl ester bond of PL to release lyso-phospholipids (lyso-PL) and free fatty acids (FFA). Additionally, this enzyme can also show PLA_2, lyso-PLA activities, as well as monoacylglycerol (MAG) and diacylglycerol (DAG) lipase activities, namely neutral lipase activity (2). This is because PLA_1 has been considered as a descendent of neutral lipases, and several PLA_1 sequences show substantial sequence similarity to pancreatic, hepatic, and endothelial lipases (7).

Although there are a number of sources of PLA_2 commercially available at various levels of purification, the fact is that PLA_1 is not yet widely accessible. This is mainly due to the difficulty in producing and purifying the enzyme, especially at industrial scale. In addition, the same PLA_1 activity is present in other lipases of different origin much more accessible, commercially available and possessing regiospecificity toward *sn*-1,3 positions, hence lipases have been traditionally used for the modification at the *sn*-1 location of PL instead of PLA_1 (4, 8). However, the availability of PLA_1 might be the overcome of low yields and conversion rates of PL at *sn*-1 location obtained with the alternative lipases. Therefore, if purified PLA_1 is achieved, it is expected to have a broad industrial application (2, 6). Specifically, PLA_1 is of particular industrial interest because it yields lyso-PL, which are excellent emulsifiers for many industrial applications, including food technology, cosmetics, and pharmaceutical industries (5).

A PLA_1 from *Thermomyces lanuginosus/Fusarium oxysporum* expressed in *Aspergillus oryzae* has been the first PLA_1 commercialized. Under the trademark Lecitase® Ultra (Novozymes A/S, Denmark), it has been mainly developed for the degumming of vegetables oils (9). More recently, a promising PLA_1 from submerged fermentation of an *Aspergillus oryzae* strain has been introduced in market for increasing the yield during cheese production under the trademark YieldMAX® PL (Chr Hansen, Denmark) (10).

2.2. PLA_2

The PLA_2 (EC 3.1.1.4) catalyzes the hydrolysis of fatty acids at the *sn*-2 position of PL, releasing lyso-PL and FFA. This is one of the most popular and well-known phospholipases as they are important proteins in signal transduction pathways in animal cells. Initially being classified in three categories, the fact is that the PLA_2 is nowadays classified in five categories as secreted PLA_2, cytosolic PLA_2, Ca^{2+} independent PLA_2, platelet-activating factor acetyl hydrolyse, and lysosomal PLA_2 (11). Additionally, the cytosolic PLA_2 can show PLA_1, lysophospholipase, and transacylase activities, and the Ca^{2+} independent PLA_2 can also acts as lysophospholipase and transacylase (1).

The use of secreted PLA_2 from porcine pancreas or snake and bee venoms has a very long tradition in food industry for modification of PL, such as egg yolk production for emulsification in mayonnaise, sauces or salad dressings, baking industry or refinement of vegetable oils by degumming (12). Thus, commercial examples of PLA_2 can be found under different trade names. Lecitase® 10 L (Novozyme A/S, Denmark) is a PLA_2 from animal origin (porcine pancreas) developed for the degumming of vegetable oils (13). Examples of microbial PLA_2 are Rohalase® MPL (AB Enzymes, Germany) or Maxapal®A2 (DSM Food Specialties, The Netherlands), this last being produced by microbial fermentation of a selected strain of *Aspergillus niger* and being mainly developed to improve emulsifying properties of egg and egg yolk. CakeZyme® and BakeZyme® are also microbial PLA_2 commercialized by DMS Food Specialties (The Netherlands) for bakery purposes.

A particular PLA_2 activity is that shown by the commercial product LysoMax® (Genencor, USA) from bacterial strain of *Streptomyces violaceoruber*. This enzyme catalyzes the selective hydrolysis of the *sn*-2 ester bond of the glycerol backbone, releasing lyso-PL for emulsifying properties. Its use in bread making has being reported (14). In oils, the LysoMax® product (LysoMax® Oil) is used to transfer the *sn*-2 fatty acid to the hydroxyl group of sterols present in the oil (lipid acyl transferase activity) during the degumming process (13).

Concerning microorganisms as source of phospholipases, the extremophile microorganisms are being especially interesting during last years, since are those microorganisms that live in harsh environments, showing similar expected stability for the own produced enzymes (6). The use of thermostable enzymes can improve the properties of industrial phospholipases and provide benefits like increased substrate solubility, decreased viscosity, and lower risk of microbial contamination (5). As example, *Pyrococcus horikoshii* or *Aeropyrum pernix* are hyperthermophilic microorganisms that produce PLA_2 with optimal activity at 90°C (6).

Other alternatives to PLA_2, different to those from pancreatic or microbial origin, are being assessed from other organisms. As example, PLA_2 extracted from starfish has shown successful results (15, 16).

2.3. PLB and lyso-PLA

A third acyl hydrolose has been described as PLB (EC 3.1.1.5), which does not discriminate between the two positional acyl ester bonds (1, 6). In addition, when partially hydrolyzed PL (lyso-PL) are the substrates of the enzyme, the lyso-PLA term is used (EC 3.1.1.5), this activity being also possible for some PLA_2, transacylases, and lipases. The lyso-PLA is less common and has not been remarked as useful for biocatalytic applications (8).

2.4. PLC

The PLC cleaves the phosphorus–oxygen bond between glycerol and phosphate, releasing DAG and phosphate esters. This enzyme is frequently classified according to specificities for the PL phosphatidylinositol (PI) (EC 3.1.4.11) or phosphatidylcholine (PC) (EC 3.1.4.3).

The industrial application of this enzyme is also limited and available just at small quantities. Cultures of *Bacillus cereus* and *Bacillus thuringiensis* are suitable for the production of the enzyme for biocatalytic applications (8). As example of commercial PLC, the microbial Purifine® (Verenium Corporation, USA) is specific for PC and phosphatidylethanolamine (PE), developed specifically for oil degumming and validated at industrial scale. Other PLC for degumming of oils has been also recently patented, such as the BD16449 PLC enzyme preparation, which is derived from a non-toxigenic, non-pathogenic, genetically modified strain of *Pichia pastoris* (17).

2.5. PLD

The PLD (EC 3.1.4.4) cleaves the phosphorus–oxygen bond between phosphate and headgroup, releasing phosphatidic acid (PA) and a hydrophilic constituent. This enzyme can also substitute the polar head of PL by transphosphatidylation in presence of a reactive hydroxyl group. Despite most PLD are capable of hydrolyzing most PL, including PC, PE, phosphatidylglycerol (PG), PI, phosphatidylserine (PS), lyso-PC, cardiolipin, and plasmalogens, the preferable substrate is PC, to generate a choline molecule and PA (2).

PLD has an outstanding position by its transphosphatidylation potential and, together with PLA_2, is one of the most studied phospholipases respect to their industrial applications (4). PLD has been used in the synthesis of PL conjugates for the use in food, cosmetic, and pharmaceutical industries (8). Therefore, PLD from different sources are commercially available. PLD from cabbage (*Savoy cabbage*) can be easily prepared from homogenates of this vegetable and can also be found in other plants such as carrots, peanuts, castor beans, or cottonseeds. Respect to microorganisms, PLD from *Streptomyces* sp. have gained importance and are also commercially available. PLD produced by fermentation of *Actinomycetes* strain has been also studied for use in industrial processes because of its higher transphosphatidylation activity than hydrolytic activity (8). With respect to recombinant production

and genetic engineering, PLD from diverse species of plants or *Streptomyces* have been obtained as recombinant proteins and are easily expressed in *E. coli* (18).

3. The Use of Phospholipases in the Industry of Vegetable Oils

3.1. Degumming of Vegetable Oils

There are many different kinds of commercial vegetable-based oils, which are separated from oilseeds and oil-bearing fruits, such as sunflower, corn, soybean, canola, rapeseed, safflower, peanut, or rice bran oils, as examples of oilseeds; and palm, olive, or coconut oils, as examples of oil-bearing fruits. Regardless of the way of extraction, vegetable oils after rendering, crushing or solvent extraction, inevitably contain variable amount of non-TAG components, such as fatty acid FFA, MAG and DAG, phosphatides, sterols, tocopherols, hydrocarbons, pigments, vitamins, sterol glucosides, glicolipids, protein fragments, and trace of pesticides and metals. Removing such undesirable impurities, which may affect quality, taste, smell, and appearance, is necessary by refining processes.

The two major purification-processing methods are chemical refining, which neutralize FFA and phosphatides with an alkaline solution, and physical refining, by distillation and steam distillation. Within these purification treatments, the degumming process is a preliminary important step. It consists of the treatment of crude oil to mainly remove phosphatides, which have a negative impact on the storage stability and downstream processing of the oil. Basically, the degumming process converts the phosphatides to hydrated gums, which are insoluble in oil and readily separated as sludge by settling, filtering, or centrifugal action.

The knowledge of the properties of phosphatides is the base for the degumming process. Phosphatides are divided in two subgroups: the hydratable phosphatides, which lastly constitute the commercial product lecithin, and the non-hydratable phosphatides (NHP). The NHP were initially described as a mixture of PA and lyso-phosphatidic acid LPA (19). PA has two hydroxyl groups and has a great affinity for divalent ions such as calcium and magnesium. Together with PA, PE has been also shown as a NHP. In fact, it seems that PC and PI are always hydratable PL, but PE and PA can be either hydratable or non-hydratable. Concerning LPA, they are more hydrophilic than its non-hydrolyzed precursor. In fact, salt of LPA will be more hydrophilic than the calcium salt of PA, but not at all LPA salts are hydratable (20).

There are different modes for removing phosphatides by degumming. The traditional degumming processes include water degumming or acid degumming. However, some of these methods are not always optimally suited for all oil qualities because of the high content

of NHP of some oils. Thus, other degumming processes have emerged, as enzymatic degumming supported by phospholipases.

3.2. Non-enzymatic Degumming

In the water degumming, water is added to crude oil at 50–75°C and at a proper amount of about 75% of the phosphatide content. The oil is mixed for 15–60 min to aid the hydratation of the PL. The easily hydratable PL forms an aggregation with a higher specific density, while NHP remain in the oil. The gum aggregation obtained is a mixture of gum and oil and may be separated by settling, filtration, or centrifugation. The hydrated gums obtained by water degumming can be vacuum dried for crude lecithin processing or recombined in meal. Water-degummed oil still contains phosphatides. Nevertheless, NHP of water-degummed oil can be made hydratable in a second step with acid degumming or by enzymatic degumming.

Therefore, the acid degumming process might be considered as a variant of the water degumming. In this process, the crude oil is mixed with acid at elevated temperatures (60–70°C). The mixture is cooled, water is added, and it is stirred for 30 min to 3 h, depending on the temperature. Finally, hydrated gums are separated by centrifugation. The acids displace the PA and PE from its calcium and magnesium salts, improving the hydrophilic nature of the NHP, and bind divalent metal ions that can be removed with the aqueous phase. Generally, industrial refineries use phosphoric acid or citric acid in degumming processes, because these two reagents are food grade and sufficiently strong, but citric acid is usually preferable because it does not increase the phosphorous content in the oil (21). Acid degumming leads to lower residual phosphorous content than water degumming. However, the lecithin gums from the acid degumming are not utilized for lecithin production, because they contain higher PA than that obtained with water degumming, as well as degumming acids (22).

3.3. Enzymatic Degumming

Enzymatic degumming consists of the use of phospholipases to modify the NHP of vegetable oil to hydratable phosphatides. The process varies depending on the phospholipase used and there are differences in the temperature and pH of the process, action on phosphatides and obtained by-products. The process comprises of two steps: (1) treating the oil with phospholipases and (2) separation of the aqueous phase containing the hydrolyzed PL from the oil.

Enzymatic degumming shows several advantages with respect to traditional degumming. Enzymatic degumming of crude edible oils is an environmental-friendly process usually carried out under mild conditions. The enzymes are highly specific, and this process reduces the amounts of acid, base, and waste during the refining process. Only 1–2% water needs to be added for efficient degumming (23). The phosphorous content is reduced to less than 10 ppm, even reaching levels as low as 3 ppm, being comparable to the

chemical process (23). Moreover, the enzymes can be re-used at least in part in the degumming process (24). An enhancement in product yield and a reduction in operating cost can also be obtained. In summary, Dijkstra (25) concluded that the enzymatic degumming processes, depending on the phospholipase, can improve oil yield, reduce possible aqueous effluents, obtain less oil in gums, and produce gums which are lecithin grade.

The industrial enzymatic degumming is quite widespread, although the published data on its efficiency is limited (26). But the fact is that the scarce published data confirms that the process can produce higher oil yields compared to traditional methods of degumming (26). Nevertheless, Dijkstra (13) suggested that, on industrial scale, enzymatic degumming processes of NHP might lead to worst results than acid degumming and that enzymatic degumming aiming at low residual phosphorous levels are to be regarded as an acid refining process that is followed by PL hydrolysis.

3.3.1. Phospholipases Used in Enzymatic Degumming

The main commercial enzymes for degumming purposes have been reviewed recently (25). Generally, PLA_1 or PLA_2 have been considered as the most relevant phospholipases in enzymatic oil-degumming, although other phospholipases have been tested, patented, developed, or commercialized (27).

The first enzymatic degumming technology developed to be used industrially was the EnzyMax® process in 1992 and based on the use of porcine PLA_2 Lecitase® 10 L, originated as by-product of insulin production (25, 28). This process was performed on an industrial scale (28) by producing a stable emulsion of crude oil with a phosphorous content, composed of PL at about 150–300 ppm, citric acid, enzyme solution, and additional water. The mixture is stirred for 6 h at 55–60°C, and the emulsion is broken in a continuously operating separator. In this step, the released lyso-PL is removed with the water phase and the enzyme adheres to the sludge formed. The majority of the sludge is brought back into the process stream, so that the enzyme can be reused several times. According to lab experiments, a minimum initial enzyme activity of 300 PLU/kg oil was necessary to decrease the phosphorous content in the oil phase to a value below 10 ppm, which was still achieved with enzyme preparations that had been used for three times (29). Additionally, the gums recovered from this process can be suitable for lecithin production (30).

As previously described, Lecitase® 10 L is an animal phospholipase from porcine pancreas. The problem of animal phospholipases is that they have a high cost and require a low dosage to be economic. Alternatively, microbial enzymes yield in a more environmentally friendly production process, have a non-limited availability, lower cost, and provide a Kosher product. Additionally, microbial enzymes reduce the dosage cost and reaction time could be shortened. Therefore, following the development of porcine

lipases, various microbial phospholipases with different specificities were developed and the porcine PLA_2 was replaced for enzymatic degumming.

The first of these microbial enzymes were the Lecitase® Novo and Lecitase® Ultra both from Novozymes A/S (Denmark) (27), which form lyso-PC and FFA. These two commercial enzymes cause a fatty acid to be split off a phosphatide, which then becomes hydratable and can be removed with the water phase(25).The aqueous mixture obtained contains lyso-PC and can be used in animal feed industry. The FFA are then removed in the deodorization step and can be used as a valuable co-product, or further processed into other products, such as biodiesel fuel (31).

In a comparative study between Lecitase® Novo and porcine Lecitase® 10 L, it was observed that the microbial enzyme preferentially hydrolyzed PL in the oil phase (lipophilic), whereas the Lecitase® 10 L mainly acted in the aqueous phase (hydrophilic) (27). The Lecitase® Novo was successfully introduced at the oil mill Cereol Germany (Mannheim, Germany) for degumming of vegetable oils (27).

The Lecitase® Ultra was developed as an improvement over the previous Lecitase® Novo, the newest showing more thermal stability (13). This is a protein-engineered PLA_1 from *Thermomyces lanuginosus/Fusarium oxysporum* expressed in *Aspergillus oryzae*. It is supplied as an aqueous solution containing approximately 6.5% protein and it is an acidic lipase which exhibits maximal activity at pH 5.0 (32). Mishra et al. (9) characterized such product and found that this preparation consisted mainly of a single protein which displays both phospholipase and lipase activities. Thus, when the temperature is over 40°C, the phospholipase activity predominates and the lipase activity is partially suppressed (32). Yang et al. (33) showed that Lecitase® Ultra could be applied for the degumming of rapeseed oil and soya bean oil, since the phosphatides in the oil are easily converted by enzymatic catalyzed hydrolysis to less than 10 mg/kg within 5 h at 50°C. Additionally, the resulting gums were more fluid than those from acid degumming, which improved their handling properties. Dayton et al. (34) successfully showed that yield loss might be reduced by using Lecitase® Ultra in an installation for soya bean oil degumming with either crude or water degummed oil. Particularly, both soapstock and refining loss were significantly reduced and yield of oil increased, from 96.6 to 97.8% for crude oil and from 98.3 to 99% for water degummed oil. With the vegetable oil prices almost continually increasing, the value of this recovered oil becomes an increasingly important reason for carrying out this form of degumming (26).

Additionally, the use of Lecitase® Ultra has been extended to the own extracted gums, which allows the recovery of the retained oil (35). The gums are collected in a stirred reaction vessel at 55°C, and 200–300 ppm of the enzyme is added together with citric acid.

After 2–3 h of reaction, the gums are heated at 80°C to break the emulsion, and the oil can be recovered by centrifugation. This oil can be further processed together with the rest of oil by bleaching and deodorization, whereas the gums can be more readily mixed with the meal left over after oil extraction.

The PLA_2 Rohalase® MPL is another commercial enzyme for the degumming of vegetable oils. This enzyme is the microbial PLA_2 co-assignee of the EnzyMax® patent, showing higher optimal pH, being superior for degumming purposes, and acting at very low water content (27).

Together with PLA_1 and PLA_2, the PLC is also of interest for the degumming process of vegetable oils. Whereas PLA leads to the formation of FFA that move into the oil phase but are removed on refining, the PLC leads to the formation of DAG that stays in the oil on refining and thus increases the oil yield (13). The commercial enzyme Purifine® is a commercial PLC that catalyses the splitting off of the phosphate group from PC and PE, and from PS if present, but it has no activity with respect to PI or PA (25). Initially, the lack of effect on PA of PLC resulted in the need for a subsequent chemical degumming stage (26). Later, a method for degumming oil simultaneously with one or more PLA and one or more PLC was patented (36). This approach solved the drawback of PLC respect to PA, since PLA might be capable of hydrolyzing most phosphatides that PLC was not able to, avoiding the combination with chemical degumming step. Additionally, this approach reduced the amount of water necessary for the process.

As previously explained, LysoMax® is not strictly a phospholipase in oils; it mainly acts as a lipid acyl transferase. Like PLA_1 and PLA_2, it releases lysophosphatides, which are removed with the water phase during the separation stage of the degumming process. However, unlike PLA_1 and PLA_2, it does not form FFA, since the LysoMax® product transfers the FFA to the hydroxyl group of sterols present in the oil, leading to less/no level of FFA in the oil, causing higher yields, improved release of oil from the gum matrix and reduced wastewater. Only in the absence of sterols, FFA are formed. LysoMax® acts on all phosphatides, so it leads to low residual phosphorus content of the degummed oil (13, 37).

Despite not commercially available, a lyso-PLA have also been suggested for degumming of oils for releasing of PG, which would be even more hydrophilic than the lyso- form. These enzymes may improve the recovery from gums as they would increase the separation of gum and oil (26).

These paragraphs have shown the introduction of commercial phospholipase in degumming of vegetable oils and the continued intense research on phospholipases different to those commercially available, showing the interest on this field and that the producers regard enzymatic degumming as an attractive opportunity (26). Thus, while enzymatic degumming was initially developed for being mainly applied to soya bean and rapeseed oils, as they were

the oils having the highest PL content, nowadays, it is being extended to other vegetable oils, including sunflower, corn, linseed, rice bran, or palm oils (26).

4. The Use of Phospholipases in Bakery Industry

The overall bakery industry has undergone significant changes owing to shifting consumer trends, particularly for products providing health convenience and satisfaction. There is a relatively increasing popularity of low-carbohydrate diets, which has given a boost to the new whole grain bread products. At the same time, commercial baked products require uniform consistent performance from all of the ingredients employed to obtain quality production at the lowest costs. This is encouraging the usage of diverse substances as the help to overcome the processing difficulties, poor volume, and unacceptable quality, otherwise associated with whole grain bread manufacturing. These substances are used to influence the structural and physicochemical characteristics of the flour constituents, in order to optimize their functionality in bread making (38). Therefore, to achieve good quality, the use of emulsifier, shortenings, and enzymes is widely extended, modifying the physicochemical properties of the dough and bread. Concerning enzymes, the baking industry makes use of different types of amylases, oxidases, hemicellulases, proteases, and recently lipases and phospholipases. The blending of all these enzymes has to be precise in order to effectively and consistently deliver the desired benefits such as maintaining bread volume, crumb softness, crust crispiness, browning, and freshness. It is important to point out that among all the enzymes used in food applications, those used in bakery industry constitute nearly one-third of the market (39).

Bread is the product of baking a mixture of flour, water, salt, yeast, and other ingredients. The basic process involves the mixture of ingredients until reaching the dough, followed by fermentation and baking the fermented dough into bread. During all the steps of bread making, complex chemical, biochemical, and physical transformations occur, which affect and are affected by the various flour constituents (38). The most relevant components of the flour and, in turn, of the formed dough are the gluten and the starch. Starch and gluten undergo a series of changes, and as the result of the heat-induced changes, the typical foam structure of baked bread is formed (38). The starch granule suffers the irreversible destruction of the molecular order (gelatinization) and is a determining factor for initial loaf firmness because of its rapid retrogradation. Gluten is the component responsible for trapping carbon dioxide released during fermentation and causes the "swelling" of the mass. During the process of baking, these proteins form networks of proteins and give the final texture to the dough.

4.1. Lipids and Emulsifiers in Bread Making

Compared to gluten and starch, lipids form a relatively minor compound in wheat. They constitute 2–2.8% of dry matter in wheat flour (40). The lipids also affect to the processability of the raw material and quality of the final products. They can be divided into free lipids and bound lipids, both fractions containing either polar or nonpolar lipids (39). Approximately, half of the lipids are polar, and the ratio of polar to nonpolar lipids is of great importance in bread making because of its strong correlation with bread volume. The polar lipid fraction is mainly composed of lyso-PL, PL, and galactolipids (14). In general, lipid functionality is related to its effect on the stability of the gas cells. In this respect, the positive influence of the polar lipids is attributed to their ability to form lipid monolayers at the gas/liquid interphase of the gas cells, thus increasing the gas retention of the dough (41). In addition, the ratio of nonpolar to polar lipids and the galactolipid content are strongly correlated with loaf volume. These functions of PL in bread making are due to their amphiphilic nature.

However, due to the minority of lipids in flour, the PL fraction of flour is not enough to reach a significant effect by themselves on the properties of dough and the quality of end-products (14). Therefore, exogenous PL or emulsifiers are needed to ensure uniform quality and shelf life stability of bakery products, which are processed under highly automated conditions, distributed, and often stored for a considerable length of time before consumption takes place. Particularly, lipids have been added as emulsifiers for the production of baked goods for many centuries. These emulsifiers have different functions, such as reducing retrogradation, stabilizing bubbles from yeast fermentation leading to larger bread volume, strengthening the gluten that has entrapped the fermentation gases, and maintaining a soft crumb, thus extending the shelf life (42).

PL isolated from soybean lecithin or egg yolk are frequently exogenous PL used in bread making. Lecithin has the ability to complex with the gluten and can function as a natural bread-dough conditioner. Exogenous PL also bind with starch and form vesicles or liposomes with excellent antistalling properties. On the other hand, PL are the antisticking agents used in baking-pan release products (43). Habitual emulsifiers different from PL used in bread making are diactyl tartaric acid esters of MAG and DAG, sodium steroyl lactylate, or MAG.

4.2. Phospholipases Used in Bakery Industry

The use of lipases and phospholipases in bread making is quite recent when compared to that of other enzymes. Phospholipases and lipases are used in the modification of PL and galactolipids, both those inherent of flour and those exogenously added, to release the corresponding lysolipids (lyso-PL and digalactosyl MAG). Lysolipids contribute in a larger way like wetting agent in bread-making process, aid in handling and shortening dispersion

of baked goods, and extend shelf life of bread loaves (14). Therefore, lipases and phospholipases offer the opportunity to generate compounds with technological effects and emulsifier characteristics in the bread making, with the main final advantage of allowing the reduction or replacement of added emulsifiers in bakery products (44, 45). On the other hand, superior emulsifying properties of released lysolipids can be obtained, leading to improved dough rheological properties (46).

Among lipases and phospholipases, the fact is that the commercial enzymes used in the bakery industry are mainly lipases with phospholipase activity. Lipases have been thoroughly studied to improve dough rheological properties, such as strength and stability, produce a more uniform crumb structure, and the crumb softness is also improved. Furthermore, lipases provide an alternative for the use of chemical dough-strengthening emulsifiers. The first generation of lipases for bakery were those 1,3-specific lipases on nonpolar lipids. However, the technical and commercial benefits were limited. Thus, enzymes with much broader specificity, acting also on polar lipids, were the second generation of lipases for bakery industry (39). Hence, lipases hydrolyze the ester bonds of TAG, yielding MAG, DAG, and FFA, and they form lyso-PL at the same time. Lipase from *F. oxysporum* is a commercial example showing both lipase and phospholipase activity, being offered by Novozymes A/S (Denmark) for baking application under the trade name Lipopan F® (47). Moreover, Lipopan® Xtra has been developed recently to complement Lipopan F®®.

However, the fact is that not all lipases are equally effective at improving bread volume. On the other hand, it has been suggested that the use of phospholipases might be a better emulsifier-replacer approach than lipases and that might provide even better emulsification in the dough (5). Therefore, diverse studies about the uses of phospholipases in bread making are being developed during last years, and this can be still considered an emerging but promising field of the industrial use of phospholipases.

Most of the assays on phospholipases in bread making have been done by the use of PLA_2. In an old patent, the first attempts were performed by Inoue et al. (48), who developed a bread improver based on PLA_2 to enhance the properties of dough and bread. It was suggested that the amount of this PLA_2 might depend on the quality of wheat flour, the type of finished baked product, the method of bread making, and the proportions of the ingredients. Generally, 10–5,000 U of PLA_2 were used per kg of wheat flour. Moreover, the combination of PLA and added lecithin improved the quality of dough. This enzyme provides the dough with a suitable degree of elasticity and extensibility, and suppressed its stickiness. Furthermore, the volume of the finished product is increased, its interior has a well stretched structure in film form, and the finished product has a suitable degree of softness.

Currently, diverse commercial PLA_2 for bread-baking purposes can be found. The PLA_2 offered by DSM Food Specialties (The Netherlands) under the trademark Maxapal®A2(10,000 U/mL) has been studied by Zhao et al. (46). These authors concluded that PLA-treated dried egg yolk could increase the dough stability and starch–gluten interaction, since the gluten network was strengthened by the PLA_2-treated egg yolk.

Recently, DSM Food Specialties also launched the product CakeZyme®, a microbial PLA_2 that is added directly to the cake batter during processing and that is able to improve the emulsifier properties of egg lecithin. This allowed the reduction of the natural emulsifier use by up to 20%, and finally resulting in less baking costs and extension of cake shelf life (49). DSM Food Specialties also offer the commercial phospholipase BakeZyme® PH 800 BG, which is a lipolytic enzyme preparation produced by *Aspergillus* species to improve dough and bread characteristics.

The PLA_2 Lecitase® 10 L, initially developed for degumming of vegetable oils, has also been used in a patent for studying the combination with bile salts in bread making (50). Adding PLA to dough together with very small quantities of bile salts showed a very positive effect on loaf volume and on other bread parameters. However, both ingredients were not as effective when applied alone. Using PLA and bile salts showed at least equivalent results than adding the combination of PLA and exogenous lipids.

Another microbial-commercial PLA_2 tested on the baking market is LysoMax® product. As previously explained, LysoMax® is a commercial product of bacterial non-genetically modified strain PLA_2, acting on lecithin sublayers with conversion into 2-lysolecithins and FFA (14). It has been shown that this enzyme can be effectively used as replacer of the traditional emulsifier DATEM due to similar rheological behavior of dough, loaf volume increase and improved properties of bread crumb (16). Another example is the product commercialized from Millbo Spa (Italy), which offers the phospholipase M300LF. In a dosage of 5–60 mg per Kg of flour, this enzyme might contribute to increase in the bread volume, which might partially or fully replace the addition of emulsifiers.

The use of a PLD for bakery purposes was also mentioned in the patent of Inoue et al. (48). It was suggested that this enzyme might be used in combination with PLA_2 to improve the properties of the bread obtained.

As example of other interesting trends, Borch et al. (51) have patented polypeptides with improved properties for making bread and other baked products by altering the amino acid sequence of a fungal lipolytic enzyme. These polypeptides have shown phospholipase activity, particularly hydrolytic activity towards ester bonds in polar lipids and showed improvement in the crumb structure, the loaf volume, and the dough stability.

On the other hand, a recent patent relates the enzymatic modification of lipids contained in cereal bran to obtain lyso-PL and lysoglicolipids with lipases and other enzymes (45). It describes a method for generating functional lipids from plant material such as bran. This method generates bread or cereal products without significant impact on these finished products (color, taste, appearance, or structure) and to make it possible to increase their health and nutritional effect. Therefore, it is suggested that side stream from processing of plant material, such as cereal bran from milling, soap stocks refining vegetable oils or distiller's dried spent grain are available as a raw material to generate functional lipids that might serve as emulsifiers in different applications.

5. The Use of Phospholipases in Dairy Products

Milk is the complex biological fluid secreted in the udder of mammals, whose main constituent is water together with proteins, fat, hydrocarbons, salts, enzymes, and vitamins. Milk fat consists mainly of TAG (95–98% w/w) and lipids are present as dispersed droplets, namely milk fat globules. These globules comprise TAG and small amounts of MAG and DAG, which are localized in the globule core at the interface between the fat globule core and its membrane. The milk fat globule membrane is mainly composed of polar lipids (0.2–2% w/w) (52). Most PL (80–90%) consist of PC, PE, and sphingomyelin, and minor fractions of PS and PI also can be found (53). Despite being minor compounds, PL have a critical role in stabilizing milk fat globules against coalescence (53).

Polar and complex lipids contained in the milk fat globule membrane have different technologic roles in dairy products. As examples, in milk processed to instant milk powder by co-spray drying, PL coat the powder particles and improves the heat stability of reconstituted milk (54, 55). Ice cream is both foam and emulsion, comprising ice crystals and an unfrozen aqueous phase containing PL. In this dairy product, as well as other aerated products, such as icings and whipped toppings, PL provide higher volumes. In addition, egg yolk has been traditionally used to improve the stability of ice cream and to reduce the effect of ice crystals (56). Low-fat dairy spreads usually contain 20–60% fat, which means that the processing of stable W/O emulsions is especially critical, hence PL are used as co-emulsifiers for stabilization (55). Milk PL also influence the phase separation during butter churning and milk fat crystallization by acting as nucleating agents or crystals' growth retarder (57). On the other hand, lecithin acts as the unique anti-spattering agent in salted margarines for use in frying, since a film of PL surrounding the water droplets prevents coalescence (55).

The biocatalyzed modification of milk fat is becoming more attractive as the number of efficient and specific commercial lipolytic enzymes is increasing. Lipases and phospholipases are used in the processing of several dairy products for improving the fat stability or increasing the yield of the elaboration process of cheese, butter, milk, and ice cream. Traditional applications of lipases include the enhancing and flavoring during the ripening of cheese or the production of lipolyzed milk fat for use as flavors in butter, coffee, cheese, or chocolate (58). In addition to reactions of lipase-catalyzed hydrolysis of milk fat, several other applications, such as interesterification or phospholipase-catalyzed modifications, are technically mature and very promising to diversify milk fat functionalities and uses (54). However, the use of phospholipases in dairy products can be considered emerging but with interesting findings according to diverse experiences, assays, and patents, as shown in the following paragraphs.

The main group of enzymes specifically involved in the hydrolysis of ester bonds in PL and assayed in dairy products are PLA_1, PLA_2, and PLB. The released lyso-PL have better oil in water emulsification properties than the corresponding PL, showing improved heat stability and being less sensitive to flocculation by Ca^{2+} and Mg^{2+} (55). PLC and PLD have been also suggested for producing interesting modifications in polar lipids of dairy products (59).

5.1. The Use of Phospholipases in Cheese Production

The traditional use of lipolytic enzymes in cheese industry has been mainly related to lipases for enhancing of flavor and ripening process, being included as components of the ripening systems (58). However, a great rise in the interest about phospholipase modification in cheese industry has taken place in recent years with a different purpose. The major focus point in optimization efforts in the cheese industry for many years has been the retention of dry-matter constituents in milk. Several approaches have been developed to increase the cheese yield, ranging from optimization of production equipment designs to alternative approaches, such as concentration of the cheese milk by microfiltration or ultrafiltration, and inclusion of whey protein (60). A special interest toward microbial phospholipases for milk polar lipid modification emerged when it was shown that a partial hydrolysis of PL increased cheese yield. This improvement is due to a higher fat and moisture retention in the cheese curd. Normally, 85–95% of milk fat and 75% of milk protein are entrapped in the cheese curd. However, the rest is lost in whey and brine during molding and stretching process. The mechanism of yield improvement due to phospholipases could be explained due to better O/W emulsification of lyso-PL than PL. On the other hand, the interaction of lyso-PL with proteins has been also proposed (10). This process increases the fat retention considerably during cheese making and improves the yield in a range of 0.7–3.8% in full-scale production of pasta filata cheese types, such as mozzarella and provolone (10, 61).

Several reports have shown the use of PLA_1 and PLA_2 in cheese production based on these promising results. Part-skim mozzarella cheese was produced from milk treated with fungal PLA_1 from *F. venenatum* (61). The PLA_1 was added prior to renneting and hydrolyzed the major milk PL (PE and PC), although this enzyme exhibited no activity toward sphingomyelin. The released lyso-PL were retained in the curds in higher amounts compared to native PL. The authors suggested that further yield improvements might be obtained by combining the use of phospholipase with enrichment of cheese milk with buttermilk PL to increase the amount of lyso-PL in the curd.

Novozymes A/S (Denmark) proposed the general use of PLA_1, PLA_2, and PLB to pretreat milk fat during milk, cheese and cream production processes (54). Ch Hansen A/S and Novozymes A/S introduced in 2005 the commercial product YieldMAX® PL. This PLA_1 enzyme solution, produced by submerged fermentation of an *Aspergillus oryzae* strain, increased the yield of mozzarella and pizza cheese production (60). The use of this enzyme seems to increase the yield of cheese more than 1% without degrading the quality of cheese, and it might lead to a better economy for industrial cheese manufacturers (10). The environmental impact of using the industrial phospholipase YieldMAX® PL in mozzarella production was successfully assessed (60). The environmental impact caused by phospholipase production was smaller compared with the savings obtained by reduced milk consumption for mozzarella production and, in turn, the use of phospholipase as a yield improvement factor is a means of reducing environmental impact of mozzarella production. This was explained by the fact that a small quantity of phospholipase saves a considerable quantity of milk (60, 62).

A similar enzyme treatment with the group of PLA_1, PLA_2, and PLB of cheese milk was also patented, significantly enhancing fat stability of cheese and cheese milk, besides providing a method for increasing the yield in cheese production (63). It has been also shown that when cheese is produced with a fat content of 5% less fat in dry matter (low-fat cheese), the cheese yield still increased, thanks to the treatment with the group of PLA_1, PLA_2, and PLB (64).

Apart from PLA_1, PLA_2, and PLB, the use of PLC and PLD has been also suggested in cheese production. A recent invention related to a method for producing cheese by treating the cheese milk with PLC from *Bacillus cereus* and/or PLD from *Streptomyces chromofuscus*, obtaining PE and PC depletion. The PLC and/or PLD, added in suitable amounts, might decrease the oiling-off effect in cheese and/or increase cheese yield (65).

5.2. The Use of Phospholipases in Milk, Condensed Milk, and Powder Milk

Fouling is a challenge for the dairy industry caused when caramelized proteins separate from milk and milk-based products during heat treatment, forming deposits on the surface of plate heat exchanger and resulting in excessive downtime and high consumption

of cleaning chemicals. The overcoming of the problem of fouling of thermal-treated milk has been recently suggested as a potential application of phospholipases. In this sense, Danisco A/S (Denmark) launched the product FoodPro® Cleanline. This enzyme is an acyl transferase that catalyses the transfer of acyl groups from the *sn*-2 position of PL to cholesterol of milk, leading to lyso-PL and cholesterol esters (66). These lyso-PL are water dispersible and have a high surface activity. Thus, milk treated with this enzyme has lower surface tension measured against a stainless steel surface than untreated milk. Through full-scale factory trials, it was shown that it is possible to reduce the amount of fouling with this acyl transferase (66). Moreover, this enzyme might reduce the trend to creaming, due to the improved emulsion stability. Interestingly, Soe et al. (66) stated up to 80% reduction in the amount of free cholesterol to form cholesterol esters in the resulting milk after the treatment with this product.

The application of phospholipases has been also assayed in condensed milk and milk powder in a current patent. These products can be elaborated from milk treated with PLA_1, PLA_2, and PLB, achieving several benefits in the process. In the treated milk, heat stability of the milk protein was better by enzyme treatment and a better emulsion stability of the fat was also found. On the other hand, less trend to fouling of dairy equipment was shown, which would improve the economy of the process (67).

5.3. The Use of Phospholipases in Other Dairy Products

Phospholipases in butter and dairy spreads have been also studied. Similarly to milk powder and condensed milk, butter might be produced from milk treated by PLA_1, PLA_2, and PLB, as described in the same patent (67). In the invention, cream is treated with phospholipases before it is used for production of butter or dairy spread, butter yield of enzyme-treated samples was higher than butter yield of control samples.

In salt-free or low-salt margarine, the standard lecithin does not function as anti-spattering agent correctly; therefore, 0.1–0.3% PC-enriched fractions with different PC/PE ratios or enzymatically hydrolyzed lecithins are applied (68). Phospholipases can be used to solve this problem, as it was shown in a patent which used the commercial Lecitase® 10 L. Thanks to this approach, lyso-PL, in contrast to PE, are not flocculated by the calcium ions from the water and the milk proteins, so they will remain active as anti-spattering agents.

A method for treating ice cream mix ingredients with the commercial PLA_1 YieldMAX® PL has been also currently patented (69). In the process, phospholipase hydrolyses at least 10% (w/w) of the PC and/or 10% (w/w) of the PE. The results indicated that taste, texture, and melting stability of the ice cream were improved. Such treatment may reduce the need for addition of emulsifiers and/or stabilizers.

6. The Use of Phospholipases in the Industry of Egg Yolk Emulsifiers

A great diversity of processed-food industries needs the use of emulsifying agents for enhancing the formulation of products, most of them being chemically derived emulsifiers. However, the natural surfactant PL lecithin is one of the most traditional and important emulsifiers for food industry. Some examples have been already described in previous sections concerning bakery or dairy products.

The major natural sources of PL derivatives for food processes are egg yolk and vegetable oils, mainly soybean oil. Gums extracted from soybean oil or vegetable oils after degumming processes are the main sources of commercial lecithin. On the contrary, lecithin extracted from egg yolk is not as commonly used in foods as soybean lecithin because of the commercial availability, its use being more specific for certain food applications, such as mayonnaise or sauce industries. In fact, soybean lecithin and egg yolk lecithin are two products clearly different. Concretely, yolk lecithin contains relatively more saturated fatty acids than soybean lecithin, leading to better oxidative stability. On the other hand, soybean lecithin creates a more stable emulsion than do egg yolk lecithin (70). Nevertheless, the emulsifying activity of egg yolk can be improved by modifying the egg yolk PL. Such enhancing of the emulsifying properties of egg yolk is the main reason of application of phospholipases in the industry of egg yolk emulsifiers.

6.1. Modification of Egg Yolk by Phospholipases

The consumption of liquid, frozen, and dried egg products has risen significantly in recent years because of their versatile applications in the food industry. Both the whole egg and egg yolk are used in baking, pastries, and mayonnaises, and inclusion of egg yolk is performed in delicatessen products or liqueurs. Particularly, egg yolk is an effective emulsifying agent for food products such as mayonnaise and bakery products due to emulsifying, tenderizing, binding properties and equally they can entrap air in certain conditions.

Egg yolk is a complex emulsion containing 50% water, 32% lipids, and 16% proteins (47). Egg yolk contains approximately 9% PL, 73% as PC, and 15.5% as PE. Most of these PL are found, together with apo-proteins, in the surface layer that surrounds the core of neutral lipids of the typical low density lipoproteins (LDL) of the plasma fraction of yolk. Precisely, such spherical LDL particles are considered mainly responsible for the emulsifying activity of egg yolk. Thus, any modification of the PL of these particles leads to modification of the structure and features of the LDL, such as adsorption, conformation, and/or elastic properties of protein layers at the O/W interface, which in turn changes the emulsifying properties of egg yolk.

Enzyme-catalyzed hydrolysis of lecithin has been implemented in industry in order to improve the emulsifying activity of egg yolk, modifying its emulsification properties, heat stability, and viscosity. The following paragraphs review some examples of applications of phospholipases for modification of PL on one of the most typical food products of such approach, namely mayonnaise.

The commercial enzyme Lecitase®10 L has been evaluated to improve the functional properties of egg yolk for mayonnaise production (71). The stability of the mayonnaise prepared with a lower proportion of modified egg yolk was greater than the mayonnaise prepared with a slightly higher proportion of non-treated egg yolk. In addition, no significant differences were detected in overall acceptability or in the perceived intensities of umami, nutty flavor, sourness, oiliness, and rancid flavor, as compared to the mayonnaise prepared with non-treated egg yolk. Therefore, this method was suggested for the formulation of mayonnaise-like emulsion products with improved emulsifying properties and dispersability, as well as lack of undesirable sensory qualities.

As previously explained, the commercial Maxapal® A2 is a PLA_2 used to obtain lysolecithin as emulsifier, being successfully used in bakery industry. Additionally, these enzymes have been also assayed for mayonnaise and sauce industries. Thanks to this preparation, a conversion of at least 85% of the egg yolk PL into a stable lyso-PL is considered as sufficient to improve the emulsifying properties. Like-mayonnaise emulsions and sauces made with Maxapal A2-treated egg yolk are heat stable up to 80°C, as opposed to 60°C for untreated egg yolk. Therefore, as advantage, these sauces could be readily pasteurized.

Interestingly, a special mayonnaise using egg yolk treated with PLA_2 has been developed (72). Mayonnaise was prepared using DAG oil. DAG oils are of current interest for nutritional and health properties, because compared with other edible oils in the form of TAG, the metabolism of DAG is rather different and seems to lead to less accumulation of body fat and reduction of body weight. In this study, the use of non-treated egg yolk and PLA_2-treated egg yolk as emulsifiers was compared for enhancing the preparation of the health mayonnaise. The results showed that the stability of mayonnaise with treated egg yolk was improved, allowing the right formulation of mayonnaise with the health DAG oil.

In addition to PLA, PLD can be also used in egg yolk modification. A PLD from *Streptomyces chromofuscus* was investigated with respect to its effect on rheological and emulsifying properties of egg yolk (73). This enzyme may be an alternative to application of PLA_2 in the food industry, because a bitter taste for such a PLA_2-treated egg yolk caused by liberated unsaturated fatty acids has been reported (74). However, using egg yolk lecithin as substrate of PLD forms mainly choline and PA, avoiding the drawback of FFA. The emulsions prepared with PLD-treated egg yolk indicated

better emulsifying activities compared to the application of untreated egg yolk. Hence stability after heat treatment of the emulsions could be significantly improved by the application of egg yolk treated with PLD. The use of PLD also led to an increase in viscosity of egg yolk, and a similar effect has been observed for PLC, which has been attributed to alterations of interactions between apoproteins and PL of the egg yolk (73).

The process for obtaining a PLD-treated egg yolk for using in foodstuffs with an effective amount of enzyme of the genus *Streptomyces* was also patented (75). The properties of egg yolk were modified to add this emulsifier to preparations of foodstuffs that particularly require improved heat-gelation properties, such as higher gel strength and emulsifying properties, which include, meat-processed foodstuffs, sponge cakes, tarts, biscuits, cookies, ice creams, doughnuts, mayonnaises, dressings, egg sheets, or crepes.

7. The Use of Phospholipases for Producing Bioactive or Functional Phospholipids

The current development of biocatalysis is being intensively studied to produce ingredients that provide a health benefit beyond the nutritional function, including prevention against illness and chronic and degenerative conditions. Concerning PL, several modifications using phospholipases can be developed to form compounds of bioactive interest, either hydrolyzing PL bonds or linking other interesting molecules to the different positions of the PL backbone.

The hydrolysis of PL by phospholipases releases diverse molecules such as PA, lyso-PL, and LPA which are molecules of bioactive interest. Likewise, these molecules might be potential lipid delivery systems of bioactive compounds when they are linked with substances related to health properties, including either bioactive fatty acids or even more atypical compounds, such as vitamins or phenolic substances. Therefore, the creation of novel molecular species of PL with added-value characteristics can be achieved, thanks to the action of phospholipases.

On the other hand, another interesting application of phospholipases in modification of PL for nutritional of health purposes, is the enrichment, purification, or obtaining of particular species of PL for which an appropriate natural source is not available (4).

The following examples illustrate some interesting modifications of PL with these described approaches. Despite that most of these applications are not developed at food industry scale at the moment, the fact is that due to the current interest on functional foods, bioactive ingredients or health foodstuffs, the potential of phospholipases for such purposes is really promising.

7.1. The Use of Phospholipases to Produce Bioactive-Breakdown Products of Phospholipids

PA is of current interest because it has been implicated in various cellular processes, in signal transduction, membrane trafficking, secretion, and cytoskeletal rearrangement. The effects of PA have been linked to the survival, proliferation, and reproduction of cells or organisms (78). The hydrolysis of egg yolk PC to release PA has been described using PLD obtained from cabbage extract or from *Streptomyces chromofuscus* (79). In a USA patent, a lipid mix with PA to treat diseases as withdrawal syndromes or cancer was prepared. PA was orally administered and it was also formulated for parenteral and topical administration (80).

LPA has been shown to act as an important intermediate in transmembrane signal transduction processes, as a platelet activating factor and in the stimulation of cell proliferation (81). Recently, it has been shown that LPA seems to be implicated in tumor cell motility and to protect and rescue intestinal epithelial cells (82, 83). Aoki et al. (81) produced LPA from PL. First, PA was released by a PLD, and then the fatty acid was hydrolyzed by PLA_1 or PLA_2 to obtain LPA. Additionally, LPA with different fatty acids were produced, since it seems that LPA exhibit differential biological activities depending on the esterified fatty acid, as a result of differential activation of the LPA receptors.

Another interesting breakdown product of hydrolysis of PL is lyso-PL. Despite the technological interest on lyso-PL as emulsifiers, these are molecules that occupy an important function in PL metabolism. They are generally considered as lipid second messengers that evoke a variety of biological responses, including immune response, platelet aggregation, cell proliferation, and differentiation (76). On the other hand, the intake of lyso-PL affects the absorption of certain nutrients and can be even used to improve it, since they have superior emulsification properties than PL due to the formation of smaller micelles in the intestinal track (77). As it has been shown throughout this whole chapter, diverse phospholipases are able to release lyso-PL, including PLA_1, PLA_2, and PLB, in processes such as degumming of oils or egg yolk modification. Additionally, an important number of examples in the scientific literature illustrate the variable reaction mediums and conditions that can be used on hydrolysis of different PL for production of lyso-PL (1, 4).

7.2. The Use of Phospholipases to Produce Non-abundant Phospholipids

Another interesting modification of PL by phospholipases is the enrichment, purification, or obtaining of particular species of PL for which an appropriate natural source is not available. In fact, most of the natural PL might be synthesized enzymatically from the major PL (4).

One example is PS. This PL has shown useful bioactive properties. It is well-known that PS is an activator of protein kinase C and it regulates the activities of various enzymes such as Na^+/K^+-ATPase (84). In clinical trials conducted in the USA and Europe, it was

indicated that PS supplemented in the diet plays important role in the support of mental functions in the aging brain (85). However, the fact is that the availability of PS from natural sources, such as animal brain, is really limited (4). Therefore, easy alternatives as the transformation of abundant PL to PS by phospholipases are developed. As example, the conversion of PC to PS using PLD preparations of *Streptomyces* sp was described (86, 87).

Cardiolipin is a complex PL with the structure of diphosphatidylglycerol. Cardiolipin is also of current interest due to attractive bioactive properties. This PL is the most characteristic of mitochondria. Biochemical studies provided evidence for the importance of this PL in the structure and function of various mitochondrial protein complexes (88, 89). While there is no direct evidence to support a causative relationship between altered cardiolipin content and a particular disease state, diverse experiments indicated a correlation between cardiolipin content and aberrant cell function (88). Although scarce information, it is interesting to point out that, as the research on cardiolipin continues and even a potential interest on supplementation with this PL in diverse circumstances might emerge, the tool for production of cardiolipin by phospholipases seems possible. Thus, PLD from cabbage can be used to synthesize cardiolipin from two phosphatidylglycerol molecules in a two-step process, starting with PLD hydrolysis of one phosphatidylglycerol molecule into PA and glycerol, and followed by PLD transphosphatidylation on the second molecule of phosphatidylglycerol, with PA as the acyl acceptor (90).

7.3. The Use of Phospholipase to Produce Phospholipids with Bioactive Fatty Acids

The consideration of the particular intestinal hydrolysis of oral PL is crucial for taking advantage of the potential bioactivity of the linked fatty acids to PL. In the lumen of small intestine, the dominant digestive enzyme for PL, namely PLA_2, hydrolyzes fatty acids from *sn*-2 location, releasing FFA and lyso-PL, with a fatty acid remaining at *sn*-1 location. Both hydrolysis products are taken up by mucosal cells and are re-secreted within chylomicrons as newly formed PL or TG (91). Therefore, it may be easy to think that linking fatty acids of bioactive interest at *sn*-1 position of PL might be of interest on the development of functional lipids in the form of PL for oral administration.

Several studies have been developed to modify PL by incorporation of fatty acids by alcoholysis, acidolysis, or esterification (92, 93). Enzymatic methods to incorporate specific fatty acids such as medium-chain and long-chain saturated fatty acids, heptadecanoic acid, caprylic acid, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and conjugated linoleic acid (CLA) into PC using mainly lipases and, at minor extend, phospholipases have been reported, as illustrated in the following paragraphs.

The health properties of *n*-3 fatty acids are well known. A regular consumption of small amounts of *n*-3 fatty acids may provide bioactive

properties such as a reduction of coronary and cardiovascular disease. On the other hand, it has been shown that *n*-3 fatty acids intake leads to the increase of PUFA-containing PC in blood. PUFA-containing PC or lyso-PC in cell membrane has been related to diverse bioactivities, such as decrease of plasma lipids or inhibition of tumor cells (94). Moreover, it was suggested that PC containing PUFA at *sn*-2 position caused changes in recombinant HDL structure, as well as influencing the fluidity and hydration of the PL environment (95). Additionally, the *sn*-2 PUFA-containing PC in blood seemed to be a good source of entry of PUFA into the brain (96). On the other hand, lyso-PC containing DHA was better captured by the brain of intravenously administered rats than lyso-PC containing other fatty acids such as C18:1, C18:2, and C20:4 (97). According to these findings, the production of PL esterified with *n*-3 fatty acids might be of great interest and might be effectively obtained by the use of phospholipases. The enrichment of lecithin with *n*-3 fatty acids by acidolysis using immobilized PLA_1 Lecitase® Ultra (Novozyme) has been reported (98). Lecithin has been also linked to *n*-3 fatty acids by PLA_2 Lecitase 10 L (Novozyme), a yolk suspension being previously hydrolyzed, and the *n*-3 fatty acids being later linked to the released lyso-PL. Other diverse examples of PL esterification with *n*-3 fatty acids by PLA_2 can be found in the scientific literature (97, 99). The esterification of lyso-PL by linking DHA at *sn*-2 position has been also reported with the use of PLA_2 (99).

CLA is the chemical term used to describing the isomers of the linoleic acid that contain conjugated double bonds. These isomers received attention several decades ago because of their diverse bioactivity, such as anti-obesity or anti-cancer effects showed in diverse studies. The CLA isomers are mainly found as TAG of milk and dairy products, as well as meat and meat products from ruminant animals. However, the average daily intake consumption of CLA from natural sources is considered relatively low to achieve the bioactive effects of CLA. Therefore, strategies for enriching or increasing the level of CLA in foodstuffs are currently under intense research. The production of synthetic lipids containing CLA different from acylglycerides, such as PLs, might be an interesting approach. Thus, a report on incorporation of CLA within PL has been described using Lecitase® 10 L PLA_2 (100).

7.4. The Use of Phospholipase to Produce Phospholipids with Other Bioactive Compounds

7.4.1. Vitamins

Vitamins are well known due to their nutritional value, together with relevant bioactivities and even potent antioxidant activity for many of them. However, their inclusion in specific foods is limited when the molecule shows an unbalanced lipophile/hydrophile nature according to the properties of the food matrices.

As example, alpha tocopherol and its homologs are widely used as antioxidant additives in a variety of foods, including edible oils and oil emulsions. However, they do not always provide effective

protection against oxidative rancidity in foods, due to the unbalanced lipophile/hydrophile ratio. The synthesis of a water-soluble vitamin E derivate from PC by PLD has been attempted (101, 102), the product showing higher affinity for PL membranes and higher activity in improving the oxidative stability of lard than the own alpha-tocopherol. Therefore, these water-soluble vitamin E derivates might be more applicable for a wide range of water-containing foods.

L-Ascorbic acid is one of the water-soluble antioxidants in the defense system against active oxygen in the interface area of membrane lipid and aqueous phase. If L-ascorbic acid is introduced as a polar head group of PL in lipid bilayer of membranes, it may exert an excellent antioxidant activity against the peroxidation occurring at membrane surface. In this sense, Nagao et al. (103) synthesized 6- phosphatidyl-L-ascorbic acid by PLD, which showed better antioxidant effect than the own L-ascorbic acid.

Other water-soluble vitamins can be modified by phospholipases, the PL-vitamin derivate being a lipophilic form of water soluble vitamins. Thus, PLD was used to catalyze the transfer reaction of the dipalmitoylphosphatidyl residue from 1,2-dipalmitoyl-3-*sn*-PC to thiamin, pantothenic acid, riboflavin, and their derivatives in a biphasic system (104).

7.4.2. Phenolic Compounds

Transphosphatidylation by the use of PLD is an effective reaction for the application of phenolic compounds in a wide variety of fields such as the production of fine chemicals or functional foods. Phenylalkanols, such as tyrosol and hydroxytyrosol (HT), are well known as natural phenolic antioxidants and have been related to bioactive properties. Within other phenols, HT has been pointed out as responsible for the health benefits associated with habitual consumption of virgin olive oil. It has been found to protect cells against oxidative stress, to reduce risks connected with aging pathogenesis, and to be active against microbial attack or cancer proliferation (105). Derivatives of HT with a better lipophile/hydrophile balance are being assayed for their potential use as antioxidants in foods and, at the same time, for the increase of bioavailability (106). In this sense, tyrosol and HT have been recently included in PL by PLD from *Streptomyces* sp (107).

Terpenes are functional compounds with an isoprenoid structure, found in the essential oils of plants. Perillyl alcohol is a naturally occurring cyclic monoterpene that has been related to apoptotic effects on prostate cancer cell lines and angiogenesis inhibitory activity (108). Interestingly, synthetic phosphatidylated monoterpenes alcohols catalyzed by PLD (phosphatidyl-perillyl alcohol, -myrtenol, and -nerol) showed a markedly antiproliferative effect on human prostate PC-3 and human leukemia HL-60 cell (109).

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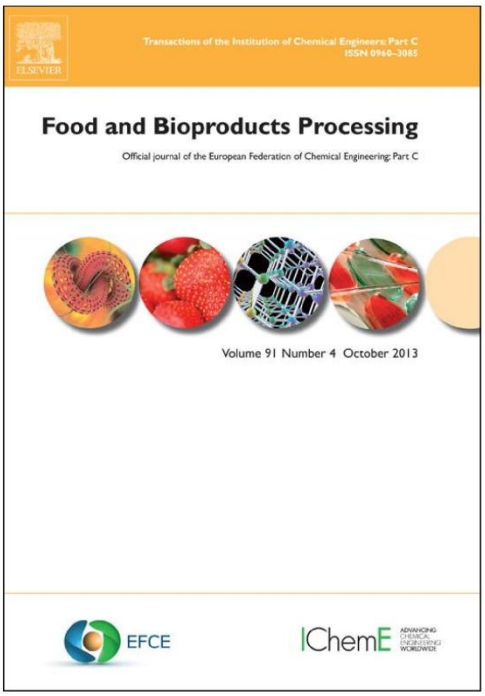

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Production and Scale-up of phosphatidyl-tyrosol catalyzed by a food grade phospholipase D

Víctor Casado[a,*], *Guillermo Reglero*[a,b], *Carlos F. Torres*[a]

[a] *Departamento de producción y caracterización de nuevos alimentos. Instituto de Investigación en Ciencias de la Alimentación (CIAL) (CSIC–UAM), C/Nicolás Cabrera n°9, Universidad Autónoma de Madrid, 28049 Cantoblanco, Madrid, Spain*

[b] *Imdea-Food Institute, CEI (UAM-CSIC), C/Faraday 7, 28049 Madrid, Spain*

A B S T R A C T

Highly purified phosphatidyl-tyrosol was obtained by using a food grade phospholipase D from *Actinamadure* sp. transphosphatidylation in a GRAS (Generally Recognized As Safe) biphasic medium. The reaction medium, comprised of an aqueous phase and ethyl butyrate, has been considered as an alternative to other biphasic systems previously reported utilizing more harmful organic solvents. The purpose of the present study was to purify phosphatidyl-tyrosol from a transphosphatidylation reaction mixture by using a procedure readily scalable to obtain a new valuable food ingredient. Initially, phosphatidyl-tyrosol was purified via semi-preparative HPLC equipment to be used as analytical standard. The best results of the transphosphatidylation reaction were obtained for two different PC concentrations, namely 83 and 166 mmol/L, with PC conversion of ca. 97 and 94% (w/w) and a final phosphatidyl-tyrosol concentration of 81 and 157 mmol/L, respectively. Finally, the procedure was scaled-up and 40 g of highly purified phosphatidyl-tyrosol (97% (w/w)) were readily purified by centrifugation without involving the utilization of organic solvents.

Keywords: Transphosphatidylation; Phospholipase D; Purification; Tyrosol; Lipophilization; Phospholipid

1. Introduction

Biocatalysis is being intensively studied to produce ingredients that provide health benefits beyond normal nutritional functions, including prevention against illness and chronic and degenerative conditions. Phospholipids (PLs) are recognized as important contributors to beneficial effects on human health, since several biological functions in cell signaling and regulation have been identified (Espinosa et al., 2011). On the other hand, PLs can be also used as emulsifiers and vehicles for transporting bioactive compounds.

Tyrosol is the most abundant phenol in extra virgin olive oil, which protect Caco-2 cells against the cytotoxic/apoptotic effects of oxidized LDL, inhibit the activity of the leukocyte 5-lipoxygenase, and improves the intracellular antioxidant defense systems. Although, tyrosol is a phenolic compound with a chemical structure unsuitable for strong antioxidant activity, it has been shown to exert powerful protective effects against oxidative injuries in cell systems when the capabilities to spare glutathione (GSH) and reinforce intracellular antioxidant defenses are considered (Di Benedetto et al., 2007). However, it should be noted that the effectiveness of tyrosol in some biological systems is related to its capability to penetrate the cells (Weitkamp et al., 2008). Moreover, in pharmaceuticals as well as in food preparations, limitations occur due to its weak solubility and stability in a lipophilic environment.

Some studies have shown that the antioxidant effects of phenolic-based antioxidants in oil matrices can be improved by lipophilization (Morales et al., 2007). Hence, lipophilic derivatives of flavonoids when esterification with aliphatic molecules increase their lipophilicity and improve their cell permeability (Fragopoulou et al., 2007). Synthesis of tyrosyl oleate as a food ingredient has also been described wherein the synthesis was catalyzed by two immobilized lipases from *Candida antarctica* in the absence of

Abbreviations: PLs, phospholipids; PT, phosphatidyl-tyrosol; PLD, phospholipase D; PC, phosphatidylcholine.

* Corresponding author. Tel.: +34 910017900.
E-mail address: victor.casado@uam.es (V. Casado).

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organic solvents. Lipophilization of tyrosol slightly improved the antioxidant activity of this phenolic compound compared to free tyrosol in oil matrices (Fernández et al., 2012).

Phospholipase D (PLD) has been extensively used as a catalyst in the transphosphatidylation of PLs in a wide variety of fields such as the production of fine chemicals and functional foods (Servi, 1999; Ulbrich-Hofmann et al., 2005). Heterogeneous reaction systems for PLD catalysis are commonly used in processes involving water-insoluble reactants. To solubilize the substrate and product at reasonable concentrations without additional surfactants, biocatalytic reactions with PLD are mostly performed in emulsion systems. In these reaction systems, the enzyme is initially dissolved in the water phase containing buffer and ions as activators (Koo and Turk, 1977; Ulbrich-Hofmann et al., 2005) with alcohol (if it is water-soluble), while the organic phase will contain the PLs and eventually the alcohol (if it is water insoluble).However, common processes make use of organic solvents that can be hazardous to human health and the environment, such as diethyl ether or ethyl acetate. Transphosphatidylation with organic solvents, up to 20 mM PC and several phenolic compounds, such as phenylalkanols, monoterpenes and other aromatic phenols, has been described (Takami et al., 1994; Yamamoto et al., 2008a, 2011). When transphosphatidylation of several primary alcohols with 2:1 (v/v) water:organic solvent ratio is employed, efficient reaction can be obtained with 200 mM PC concentration in the organic phase, but the purification of these compounds is not straightforward (D'Arrigo et al., 1996). Although there are bio-catalysis methodologies, which avoid using toxic solvents or generation of toxic byproducts, they yield low amounts of the modified PLs (Juneja et al., 1992). In this sense, transphosphatidylation of other alcohols in a solvent free medium has been already investigated (Iwasaki et al., 2003). In this mentioned work, an aqueous suspension system comprised of a solution containing serine, PLD, and lecithin adsorbed on silica or calcium sulfate powder at 40 °C was utilized. The reactant concentration was PC 10 mM, serine 1.48 M in the presence of 3 Units of a PLD from a recombinant strain of *Escherichia coli* bearing the PLD gene of *Streptomyces antibioticus* (One unit of PLD activity was defined as the amount of enzyme that hydrolyzes 1 mol PC in 1 min at 37 °C). This reaction generated more than 80% of phosphatidylserine in 24 h. However the concentration of PC adsorbed on silica is quite low, thus this methodology is only able to produce low concentrations of phosphatidylserine. Alternatives to the emulsion system are membrane reactors, use of immobilized enzymes, and an aqueous phase for the production of phosphatidylglycerol (Servi, 1999).

Regarding isolation of the new PL synthesized directly from the reaction system, temperature and salt concentration of the reaction mixture can affect phase behavior and consequently the partitioning coefficient of remaining substrates, products and protein. It is noteworthy that traditionally PL isolation has been accomplished by using acetone precipitation and solvent fractionation in combination with column chromatography.

Therefore, the enzymatic transphosphatidylation avoiding the use of harmful organic solvents for foods purposes to produce modified PLs containing a molecule of tyrosol in the polar head becomes relevant. Two main objectives need to be fulfilled: (1) an adequate procedure readily scalable to produce phosphatidyl-tyrosol (PT); and (2) an appropriate methodology for purifying the PT produced. For that matter, the present study shows an easily scalable methodology to produce highly purified PT. In addition, transphosphatidylation was carried out in the presence of a food grade PLD commercially available. Finally, in an attempt to avoid using any additional solvent in the isolation step, the purification of PT from the product mixture was attained by semi-continuous centrifugation. A schematic representation of the proposed phosphatidylcholine transphosphatidylation with tyrosol is shown in Fig. 1 and a complete flow sheet diagram of the process is depicted in Fig. 2.

2. Materials and methods

2.1. Materials

Hydrogenated phosphatidylcholine, 90% (PC 90H), was purchased from Lipoid (Cham, Switzerland). Powdered preparation of *Actinamadure* sp. phospholipase D (EC 3.1.4.4) was acquired from Meito Sangyo CO (Nikko-Cho, Fuchu, Tokyo, Japan). According to the vendor specifications PLD contained 1500 U/mg (1 PLD Unit is defined as the amount of enzyme producing 1 μmol/h of choline from L-a-phosphatidylcholine when the enzyme solution is reacted at pH 8 at 30 °C). Tyrosol (98.0% w/w purity) was purchased from TCI Europe (Zwijndrecht, Belgium). Anhydrous calcium chloride (PRS Grade), formic acid (98%), ethyl butyrate (99.5%), squalene (97%) triethylamine (99.5%), and sodium acetate trihydrate (99%) were acquired from Sigma–Aldrich (St. Louis, MO, USA). Alpha tocopherol was purchased from BTSA (Madrid, Spain), lysophosphatidylcholine (1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine) was acquired to Avanti Polar Lipids. Inc.

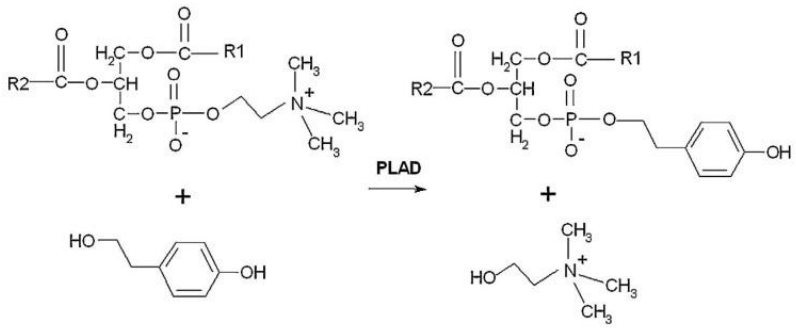

Fig. 1 – Schematic representation of phospholipase D (PLD) transphosphatidylation reaction.

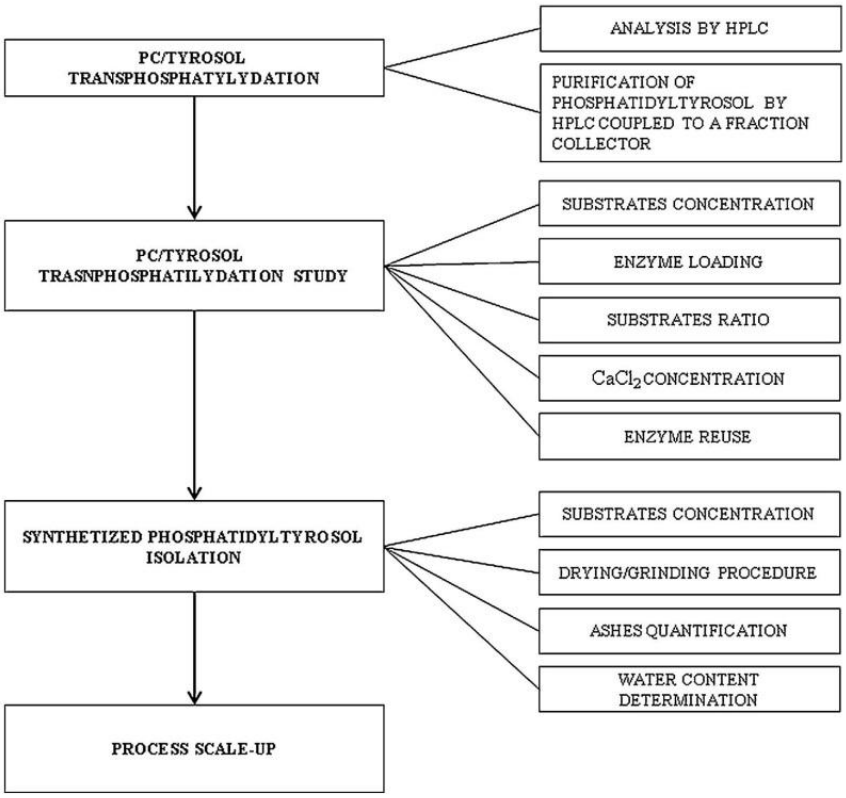

Fig. 2 – Schematic representation of the process investigated.

(Alabama, USA). Chloroform, 2-propanol, hexane, acetic acid, and methanol (HPLC grade) were purchased from Lab-Scan (Dublin, Ireland).

2.2. *Phosphatidylcholine transphosphatidylation*

Different quantities of PC and tyrosol were added to a mixture of acetate buffer and ethyl butyrate (1:2 v/v) in a 60 mL flask and mixed by swirling. The aqueous phase was comprised of 0.2 M sodium acetate buffer (pH 5.6) and 1% (w/w) of squalene (regarding total reaction mixture) as internal standard. Variable amounts of phospholipase D were added to the mixture. The flasks were placed in a temperature-controlled orbital shaker (IKA KS 4000, Staufen, Germany) at 40 °C and 200 rpm.

To improve the homogenization of the biphasic reaction mixture, commercial tyrosol was ground in Retsch mill (Grindomix, Haan, Germany) and sieved through different sieves (from 500 to 100 μm). To minimize the influence of particle size on reaction rate the transphosphatidylation took place with tyrosol below 100 μm particle size. In order to make results readily comparable with each other, all concentrations have been expressed as mmols per total volume of both organic and aqueous phase (free of humidity and mineral salts). As an example of a typical transphosphatidylation reaction, 208 mmol/L of tyrosol, 21 mmol/L of PC, 10 mmol/L of squalene, and 67 mmol/L of $CaCl_2$ were mixed with 0.8 mL of buffer and 1.6 mL of ethyl butyrate. When the mixture reached 40 °C, 1 mg of PLD (1500 units/mg) were added to start the reaction.

The reaction mixture was stopped after different time intervals and extracted with 13.6 mL of chloroform/methanol/water (8/4/1.6, v/v/v) for analytical purposes and semi-preparative HPLC purification of PT. Centrifugation separated the mixture into two phases, an upper aqueous phase and a lower phase. The lower phase was recovered and the solvent was evaporated by using N_2 until a residue with constant weight was attained, the residue was dissolved in 2-propanol/hexane 1/1 (v/v). The final transparent solution was analyzed by HPLC.

In order to determine the experimental error, some replicates of the mentioned extraction procedure were effected. In all reactions using 20.8 mmol/L PC, the standard deviation for remaining PC and remaining tyrosol was <1 mmol/L. Regarding PC conversion the standard deviation was ca. 1%.

In all reactions using 83.3 mmol/L PC the standard deviation for concentration of PT produced, remaining PC, and remaining tyrosol, was <2 mmol/L. Regarding PC conversion the standard deviation was lower than 2%.

In all reactions using 167 mmol/L PC, the standard deviation for concentration of PT produced, remaining PC and remaining tyrosol was <4 mmol/L. Regarding PC conversion the standard deviation was lower than 3%.

2.3. *HPLC analyses*

Twenty μL of the final transparent solutions were analyzed on a Luna 5 μm HILIC diol column (250 mm, 4.60 mm, Phenomenex, Torrance, CA, USA) coupled to an Agilent (Santa Clara, CA, USA) 1200 Series HPLC containing a temperature-controlled column compartment, quaternary

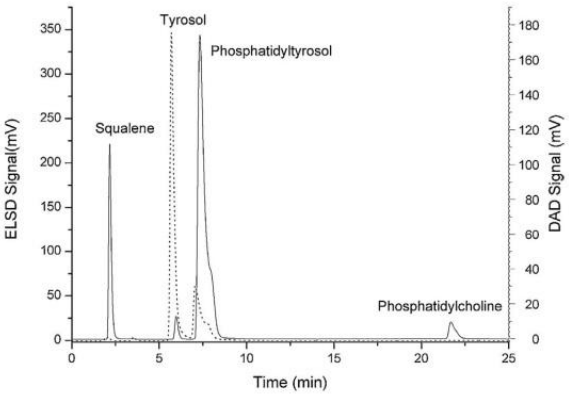

Fig. 3 – Chromatogram of a phosphatidylcholine transphosphatidylation reaction mixture.

pump, autosampler, vacuum degasser, and a dual detection system comprised of an evaporative light scattering and a diode array detector.

The flow rate was 1.5 mL/min. A splitter valve was used after the temperature-controlled column compartment and only 30% of the mobile phase was directed through the detector (3.5 bar and 41 °C). The column temperature was maintained at 55 °C. The mobile phase consisted of a binary gradient of A: hexane/2-propanol/acetic acid/triethylamine (815/170/15/0.8, v/v/v/v), B: 2-propanol/water/acetic acid/triethylamine, (837/140/15/0.8 v/v/v/v). The method starts at 3% B increasing up to 15% in 20 min. The percentage of B was again reduced to 3% in 2 min and the initial conditions were maintained for 10 min. A chromatogram of phosphatidylcholine transphosphatilydation reaction mixture is shown in Fig. 3.

Identification and quantification was carried out by using standards for each lipid class involved in the transphosphatilydation reaction. In order to minimize error when using HPLC with ELSD, rigorous calibration curves for each lipid class were developed for each set of samples injected, since the detector response was non-linear and specific to each compound.

2.4. *Purification of PT by semi-preparative HPLC coupled to a fraction collector*

A modified version of the methodology for HPLC analysis was utilized to purify PT to be used as a standard for HPLC analyses. A fraction collector coupled to the HPLC was utilized. The flow rate was 6 mL/min. A splitter valve was used after the temperature-controlled column compartment and only 10% of the mobile phase was directed through the detector (3.5 bar and 41 °C). The column temperature was maintained at 55 °C. The mobile phases described in Section 2.3 with different gradient elution were utilized. Briefly, the method started at 1% B increasing to 25% in 20 min. This percentage of B was reduced to 1% in 2 min, and the initial conditions were maintained 10 min.

Several consecutive injections of 200 μL were introduced on a semi-preparative Kromasil Sil column (5 μm, 250 mm, 10 mm), acquired from Analisis vinicos (Tomelloso, Spain) to obtain enough PT to be used as analytical standard.

2.5. *Enzyme reutilization*

A small scale-up procedure was carried out in order to improve handling the reaction products in the analytical separation step and subsequent recovery. Three reaction conditions (namely A, B, and C) were scaled-up to 24 mL of reaction medium in a 250 mL Erlenmeyer flask placed in a temperature-controlled orbital shaker at 200 rpm. The reactions conditions were: (A) 833 mmol/L of tyrosol, 167 mmol/L of PC, 55 mmol/L of squalene, 8 mL of aqueous phase, and 16 mL of ethyl butyrate with 80 mg of PLD (1500 units/mg); (B) 417 mmol/L of tyrosol, 83.3 mmol/L of PC, 30 mmol/L of squalene, 8 mL of aqueous phase, and 16 mL of ethyl butyrate with 40 mg of PLD (1500 units/mg), and (C) 208 mmol/L of tyrosol, 20.8 mmol/L of PC, 10 mmol/L of squalene, 8 mL of buffer, and 16 mL of ethyl butyrate with 10 mg of PLD (1500 units/mg). In addition, A and B reactions were repeated in the presence of variable concentrations of $CaCl_2$ (17, 34 and 67 mmol/L).

After 24 h, the reaction mixtures were introduced in a 25 mL centrifuge tubes and centrifuged at 8960 × *g*, 40 °C for 10 min. After centrifuging, two liquid phases and a solid interphase were obtained. The upper phase, comprised mainly of ethyl butyrate and tyrosol, was recovered and weighted to be utilized in a second trial. The interphase and aqueous phase were centrifuged again and separated. This aqueous phase was only partially recovered and was comprised mainly of buffer, choline, and enzyme. The volume of aqueous phase recovered for reutilizing the enzyme preparation was lower to the initial amount utilized, hence, recovered ethyl butyrate phase was added proportionally to the recovered aqueous phase to maintain the same ratio utilized in the first trial. PC was added accordingly to maintain the initial tyrosol/PC molar ratio. Both phases were mixed together with PC in a second trial to obtain a reaction mixture with the same composition to that of the first trial. The reaction mixture was heated at 40 °C and stirred at 200 rpm to start the second trial. After 24 h the mixture was extracted as described in Section 2.2 and analyzed by HPLC.

2.6. *Recovery of phosphatidyl-tyrosol and tyrosol from reaction mixtures*

A similar procedure as described for enzyme reutilization (namely reactions A, B, and C) was carried out to investigate the recovery of tyrosol and also to obtain a product comprised mainly of purified PT. In addition, A and B reactions were repeated in the presence of variable concentrations of $CaCl_2$ (17, 34 and 67 mmol/L) and after 24 h, the reaction mixtures were centrifuged as previously described.

The upper phase was first removed, but unlike the enzyme reutilization methods, the upper phase was introduced into a rotary evaporator to recover a residue of tyrosol and condensed ethyl butyrate. Then a second centrifugation with the interphase and the lower phase was performed to obtain a solid residue after removing the lower phase. The solid residue was then evaporated in a rotary evaporator and a dried PT residue was obtained, which was then ground in the Retsch grindomix mill. Finally, dried PT residue attained was analyzed by HPLC. PT composition, PC remaining composition, and tyrosol remaining composition of the mentioned dried PT residue were calculated as weight percentage. The remaining mass balance of the product was comprised of squalene used as internal standard.

The powder was analyzed as described for phosphatidylcholine transphosphatidylation. A Karl Fischer automatic titrator 870 Titrino Plus (Metrohm, Herisau, Switzerland) with hydranal composite 5 (Sigma–Aldrich, St. Louis, MO, USA) was used to determine moisture. Ash content of the PT produced was determined by using a muffle furnace. In order to determine experimental error, replicates of the centrifugation procedure were carried out. The standard deviation regarding composition of PT was 3.2%. The standard deviations of tyrosol composition, PC composition, squalene composition, and PT isolation yield were <2%.

2.7. *Scale-up of the transphosphatidylation*

Finally, the process was also scaled-up to 660 g of reaction mixture in a 1 L stainless steel reactor coupled to a paddle stirrer at 400 rpm (Kiloclave, Buchi Glass Uster, Switzerland). A K temperature probe in direct contact with the reaction mixture coupled to a heating jacket with thermal oil was used to thermostatize the reaction vessel. In addition, to prevent oxidation a N_2 atmosphere was also utilized to pressurize the reaction vessel. The centrifugation stage was also modified, and the product mixture was conducted to a thermostatized semi-continuous centrifuge (Cepa laboratory centrifuge LE, Wesseling-Berzdorf, Germany) by 2 bar Nitrogen pressure. The centrifuge was thermostatized at 40 °C and prepared to separate the liquid phases through a clarifying cylinder. Finally the solid residue inside the cylinder was evaporated as described in Section 2.6 and analyzed as Section 2.2. Fig. 4 shows a diagram of the PT production and semi-continuous separation system employed in this study.

3. Results and discussion

3.1. *Phosphatidylcholine transphosphatidylation*

A transphosphatidylation reaction between PC and tyrosol has been developed in a biphasic system containing sodium acetate buffer and ethyl butyrate, utilizing a food grade PLD from *Actinamadure* sp. as a biocatalyst. Initially, several substances such as, butyric acid, oleic acid, ethyl butyrate, triacetin, and tributyrin were added as organic phase to achieve an adequate reaction mixture (data not shown). These substances were chosen based on several criteria: (A) negligible toxicity and (B) null interference with both transphophatidylation and HPLC analyses. Usually, solvents such as ethyl acetate or diethyl ether in combination with an aqueous phase in a biphasic system are commonly utilized as an effective medium in transphosphatidylation reactions (Hosokawa et al., 2000; Takami et al., 1994). However, ethyl acetate or diethyl ether are not suitable for food applications owing to its toxicity, therefore, in the present study, enzymatic synthesis of PT in a GRAS solvent/aqueous phase biphasic medium has been considered as an alternative.

In transphosphatidylation reactions, the nature of the substrates plays an important role. The partition of substrates and products between phases, enzyme denaturation and diffusional processes determine the reaction rates (Ulbrich-Hofmann et al., 2005). In addition, the concentration of substrates and products that can be solubilized in the reaction mixture also depends on the nature of the solvents. In this respect, the best results of the present study were obtained with ethyl butyrate and it was selected for further experiments.

Ratios higher than 1:2 water:organic solvent and higher than 1:10 PC:alcohol are commonly employed (Hosokawa et al., 2000; Yamamoto et al., 2008a, 2011). Hence, in order to compare all the experiments of this work, a commonly described transphosphatidylation reaction was initially selected as a comparative model. The reaction took place using a ratio 1:2 (v/v) of buffer (0.8 mL) and ethyl butyrate (1.6 mL, containing 1500 units of PLD, PC 20.8 mmol/L, and a molar ratio 1:10 of PC:tyrosol. These reaction parameters have already been described to produce PT but using ethyl acetate as organic solvent. In the present study similar results were attained replacing ethyl acetate by ethyl butyrate.

Then several changes were introduced in the original reaction mixture to obtain the best reaction conditions regarding to PC conversion, enzyme loading and product isolation from the reaction mixture. Therefore to assess the results from the different reaction mixtures assayed various variables such as; PT concentration (Eq. (1)), remaining PC concentration (Eq. (2)), remaining tyrosol concentration (Eq. (3)), $CaCl_2$ concentration (Eq. (4)), PC conversion (Eq. (5)), and PT isolation yield (Eq. (6)) were considered. The different variables utilized were calculated with the following formulas:

$$\text{PT concentration} = \frac{\text{mmols PT produced}}{\text{L organic phase} + \text{L aqueous phase}} \quad (1)$$

$$\text{Remaining PC concentration} = \frac{\text{mmols remaining PC}}{\text{L organic phase} + \text{L aqueous phase}} \quad (2)$$

$$\text{Remaining tyrosol concentration} = \frac{\text{mmols remaining tirosol}}{\text{L organic phase} + \text{L aqueous phase}} \quad (3)$$

$$\text{CaCl}_2\text{ concentration} = \frac{\text{mmols CaCl}_2\text{ added}}{\text{L organic phase} + \text{L aqueous phase}} \quad (4)$$

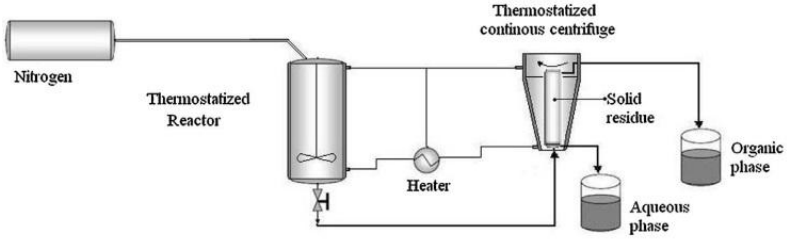

Fig. 4 – Diagram of the PT production and semi-continuous separation system.

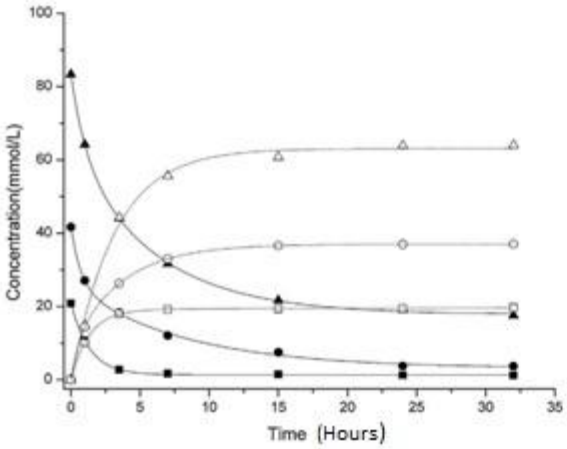

Fig. 5 – Time course of the phosphatidylcholine transphosphatidylation in the presence of different phosphatidylcholine concentrations. Concentration of remaining PC (solid line). Concentrations of phosphatidylcholine utilized: 20.8 mmol/L (■), 42 mmol/L (●), and 83 mmol/L (▲). Concentration of PT (dashed line). Concentrations of phosphatidylcholine utilized: 20.8 mmol/L (□), 42 mmol/L (○), and 83 mmol/L (△).

$$\text{PC conversion} = 100 - \left(\frac{\text{Remaning PC concentration} \times 100}{\text{Initial PC concentration}}\right) \quad (5)$$

$$\text{PT isolation yield} = \left(\frac{\text{mg powder isolated} \times \text{PC conversion}}{\text{initial PC mmol} \times \text{PT molecular weight}}\right) \quad (6)$$

At the starting reaction conditions previously mentioned PC conversion was ca. 94% (w/w). Firstly, concentration of substrates was increased (Table 1) while keeping constant the rest of reaction variables. The concentrations of PC studied in this work were 21, 42, 83, 167 and 333 mmol/L, all of them with a molar ratio 1:10 of PC:tyrosol. Good results were obtained by using 20 and 41.6 mmol/L of PC, ca. 94% (w/w) and ca. 91% (w/w) of PC conversion, however, 83.3 mmol/L of PC only produced ca. 76% of PC conversion, and PT was not attained by using 166.6 mmol/L of PC. Fig. 5 shows the time course of the phosphatidylcholine transphosphatidylation in presence of 1500 units of PLD and different phosphatidylcholine concentrations. The time required to obtain 36 mmol/L of PT was 15 h in a reaction with 42 mmol/L of PC. However transphosphatidylation reaction at the lowest concentration of PC (21 mmol/L) required only 5 h to obtain ca. 19 mmol/L of PT. Besides, high concentration of PT was achieved in a relatively short time period, 83 mmol/L of PC, produced 63 mmol/L of PT (w/w) in 15 h. Higher initial concentrations of PC such as 167 mmol/L did not produce PT in the product mixture (Table 1).

In previously published results, transphosphatidylation has been commonly studied using only low concentration of PLs (Juneja et al., 1992). A biphasic system comprised of a dissolution of PC in diethyl ether (106 mM) and a saturated dissolution of serine (3.4 M) utilizing 1 g of two PLD aqueous preparations of Streptomyces (1.71 and 0.017 (U/mg) was reported to yield only 36% (w/w) of phosphatidylserine (One unit of PLD was defined as the amount of enzyme hydrolyzing 1 μmol of pure PC per min at 30 °C). However, a reaction mixture utilizing dissolution of PC in diethyl ether (17.8 Mm) achieved 85% (w/w) of phosphatidylserine in 30 min. These results are attributed to PLD inhibition by choline at high PC concentrations. This study also indicates that choline is difficult to remove from the product mixture at the end of the reaction. It has been described, that one of the major problems associated with scaling-up the process is product inhibition at high substrate concentrations which results in decreased yields. As it has been already mentioned, the problem has been shown to be associated with the concentration of choline which tends to reverse the transesterification equilibrium. A possible solution is the addition of specific choline-transforming enzymes like choline oxidase and catalase to avoid choline inhibition, but, as it has been already stated, enzymes are the most expensive ingredients in biocatalysis, and a dual enzymatic system would increase dramatically the costs of the procedure (Juneja et al., 1992).

On the other hand, in order to study the influence of enzyme loading on the transphosphatidylation reaction, PLD was added to become 1% (w/w) of the substrates (Table 2, rows 1 to 5). In addition, enzyme loading was also increased to become 5% and 10% by weight of reactants to obtain higher conversion in reactions with high concentration of substrates (Table 2, rows 6 to 11). The PC conversion using 167 mmol/L of PC was ca. 73%, 74%, and ca. 82%, using 1%, 5%, and 10% by weight of PLD. Therefore although PC conversion was slightly higher using 5% and 10% of PLD, the improvement obtained could be considered small, and this strategy was discarded since the enzyme is the most expensive ingredient in this process. The molar ratio was subsequently investigated (Table 3). First of all, several reactions modifying the concentration of PC and keeping constant the concentration of tyrosol (208 mmol/L) were effected. Therefore, 1:2, 1:3, 1:5, 1:10 and 1:15 PC:tyrosol molar ratios were studied (Table 3, rows 1 to

Table 1 – Substrate concentration study after 24 h of transphosphatidylation reaction.

PLD mg (1500 U/mg)	Tyrosol concentration (mmol/L)	PC concentration (mmol/L)	Produced PT concentration (mmol/L)	Remaining PC concentration (mmol/L)	Remaining tyrosol concentration (mmol/L)	PC conversion (% w/w)
1	208	21	20	1	189	94
1	417	42	36	4	379	91
1	833	83	63	19	769	76
1	1667	167	0	167	1667	0
1	3333	333	0	333	3333	0

Table 2 – Enzyme loading study after 24 h of transphosphatidylation reaction.

PLD mg (1500 U/mg)/% PLD (w/w)	Tyrosol concentration (mmol/L)	PC concentration (mmol/L)	Produced PT concentration (mmol/L)	Remaining PC concentration (mmol/L)	Remaining tyrosol concentration (mmol/L)	PC conversion (% w/w)
1/1	208	21	20	1	188	94
2/1	416	42	39	2	377	94
4/1	833	83	74	9	759	89
8/1	1667	167	122	45	1545	73
16/1	3333	333	0	333	3333	0
20/5	833	83	79	42	792	95
40/10	833	83	80	33	783	96
40/5	1667	167	123	43	1543	74
80/10	1667	167	137	30	1530	82
80/5	3333	333	0	333	3333	0
160/10	3333	333	0	333	3333	0

Table 3 – Study of the influence of substrates molar ratio after 24 h of transphosphatidylation reaction with 1 mg of PLD (1500 U/mg).

Tyrosol: PC ratio	Tyrosol concentration (mmol/L)	PC concentration (mmol/L)	Produced PT concentration (mmol/L)	Remaining PC concentration (mmol/L)	Remaining tyrosol concentration (mmol/L)	PC conversion (% w/w)
2:1	208	104	83	21	125	80
3:1	208	62	52	11	156	83
5:1	208	42	38	4	171	90
10:1	208	21	19	1	189	93
15:1	208	14	13	1	196	93
2:1	83.3	42	79	25	4	76
3:1	112	36	34	8	79	81
5:1	150	32	30	6	120	84

5), total substrates concentration of these reactions were 10, 8, 6, 4, and 3% (w/w) respectively. The best results produced ca. 93% (w/w) of PC conversion using the lowest concentrations of PC (1:10 and 1:15). Obviously, very high concentration of remaining tyrosol was found in these product mixtures. However, 1:5 PC:tyrosol molar ratio could be considered as the more convenient ratio since produced 90% (w/w) of PC conversion with lower concentration of remaining tyrosol. Besides, PC to tyrosol molar ratios of 1:2, 1:3, and 1:5 were studied by changing tyrosol concentration (83.3, 112.5 and 150 mmol/L respectively) but maintaining total substrates concentration at 4% (w/w) in the reactions mixtures (Table 3, rows 6 to 8). In these reactions PC conversions were lower than those obtained in the reactions using 1:2, 1:3, and 1:5 PC:tyrosol molar ratios at 208 mmol/L of tyrosol. However the amount of remaining tyrosol is significantly lower in reactions with 4% of total concentration of substrates in the reaction mixtures. It can be concluded that at these reaction conditions, tyrosol is more efficiently utilized.

Finally 166 mmol/L of PC and 1% of PLD were used in a transphosphatidylation reaction utilizing 1:15 PC:tyrosol substrate molar ratio to study the influence of high substrate molar ratio at high PC concentration. Although, the PC conversion rate was higher than 1:5 substrates ratio, the remaining tyrosol in this reaction (2345 mmol/L) could be considered unnecessary.

The influence of $CaCl_2$ concentration in the aqueous phase of the reaction mixture was finally studied. For this purpose, the reaction with a 1:10 molar ratio, namely 20.8 mmol/L of PC and 208.3 mmol/L of tyrosol was chosen. Table 4 (rows 1 to 4) shows compositions of product mixture after 24 h of transphosphatidylation reaction carried out at the same reaction conditions but using different $CaCl_2$ concentrations (17, 34 and 67 mmol/L). It can be observed that utilization of 17 mmol/L of $CaCl_2$ was enough for achieving a ca. 99% (w/w) of PC conversion.

The final optimization study was effected to achieve high concentration of PT and high PC conversions. Addition of

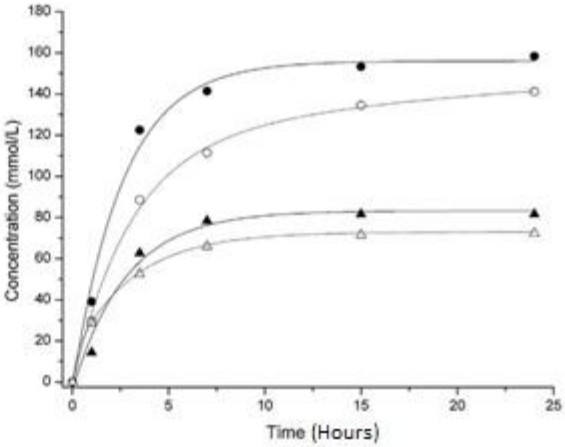

Fig. 6 – Influence of the $CaCl_2$ in the time course of phosphatidylcholine transphosphatidylation. Concentration of PT produced using $CaCl_2$ (dashed line). Concentrations of phosphatidylcholine utilized: 83.3 mmol/L (△) and 166.6 mmol/L (○). Concentration of PT produced without $CaCl_2$ (solid line). Concentrations of phosphatidylcholine utilized: 83.3 mmol/L (▲), and 166.6 mmol/L (●).

Table 4 – Study of the influence of the aqueous phase composition after 24 h of transphosphatidylation reaction.

PLD mg (1500 U/mg)	$CaCl_2$ concentration (mmol/L)	Tyrosol concentration (mmol/L)	PC concentration (mmol/L)	Produced PT concentration (mmol/L)	Remaining PC concentration (mmol/L)	Remaining tyrosol concentration (mmol/L)	PC conversion (% w/w)
1	0	208	21	19	1	189	93
1	17	208	21	21	0	188	99
1	34	208	21	21	0	188	99
1	67	208	21	21	0	188	99
4	0	417	83	71	12	348	86
4	17	417	83	71	12	348	86
4	34	417	83	75	8	342	90
4	67	417	83	81	3	336	97
8	0	833	167	142	25	692	85
8	67	833	167	157	10	677	94

$CaCl_2$ increased the PC conversion at 1:10 PC:tyrosol molar ratio and at low PC concentration (20.8 mmol/L). Hence, the same concentrations of $CaCl_2$ (17, 34 and 67 mmol/L) were also utilized in reactions with 83 mmol/L of PC and 1:5 PC:tyrosol molar ratio (Table 4, rows 5 to 8). Unlike the reactions with 20.8 mmol/L of PC, the highest concentration of $CaCl_2$ in reactions with 83 mmol/L of PC achieved a higher PC conversion, therefore $CaCl_2$ concentration is more relevant in reactions with high PC substrates. Thus reactions with 167 mmol/L of PC and 1:5 PC:tyrosol molar ratio was also studied with and without $CaCl_2$ (Table 4, rows 9 and 10). Fig. 6 shows the influence of $CaCl_2$ in the time course of phosphatidylcholine transphosphatidylation using 83 and 166 mmol/L of PC. The addition of 67 mmol/L of $CaCl_2$ produced a higher PC conversion (ca. 97% and 94% of conversion) and PT concentration (81 and 157 mmol/L) compared to that obtained without addition of $CaCl_2$. Although it has been described that Ca^{2+} is a requirement apparently no significant for PLDs from microorganism as *Streptomyces* species (Ulbrich-Hofmann et al., 2005), several transphosphatidylation reactions in the presence of plant and microorganism PLD containing 40–100 mM $CaCl_2$ dissolved in the aqueous phase have been described (Hirche et al., 1997; Mandal et al., 1980; Yamamoto et al., 2008b).

The optimal reactions conditions were chosen based on several criteria: (1) low enzyme loading to achieve a cheaper procedure, and (2) a high PC concentration. In addition, remaining tyrosol concentration was not considered as a detrimental factor, since an easy recuperation of tyrosol by rotary evaporator from the ethyl butyrate phase was achieved. Briefly, the best results were obtained with 1% enzyme loading (w/w), 1:5 substrate ratios and 67 mmol/L of $CaCl_2$. At these reaction conditions two PC concentrations namely 83 and 166 mmol/L were chosen.

3.2. *Enzyme reutilization*

Reutilization of the enzyme preparation is a key factor in bioprocessing in order to increase productivity. The purpose of this study was to recover both aqueous phase containing the enzyme, and ethyl butyrate phase containing unreacted tyrosol from the first trial to be reutilized in a second trial with a new charge of PC.

The influence of substrate concentration in the reutilization was studied at three reaction conditions (A, B, C). The composition of these reaction mixtures, the separation of the different phases by centrifugation, and the preparation of the reaction mixture for the second trial have been previously described in Section 2.5. After centrifugation, a triphasic system comprised of the aqueous phase, ethyl butyrate, and PT produced was observed. The ethyl butyrate phase, containing mainly tyrosol, was easily recovered, and 90% (w/w) of the remaining tyrosol could be obtained. However aqueous phases recovered after centrifugation from reactions (A), (B), and (C), represented only 55%, 60%, and 80% (w/w) of the original aqueous phase, respectively. It should be pointed out that the higher the substrate concentration the lower the recovery of both phases attained. The second trials were prepared at the same reaction conditions for 24 h, but these reactions achieved significantly lower conversions than the first trials. Concerning PC conversion, ca. 13%, 44% and 80% were obtained in the second trial of (A), (B), and (C) reactions respectively. Hence, only reactions with low substrate concentration seem to be appropriate for enzyme reutilization. As it was previously stated, choline concentration in the aqueous phase has been described as a reaction inhibitor (Juneja et al., 1992), thus the enzyme reutilization in transphosphatidylation requires a choline removal step. On the other hand, enzyme reuse was also studied in reactions (A and B) in the presence of 17, 34 and 67 mmol/L $CaCl_2$. The aqueous phase was easily separated after centrifugation (above 90% (w/w) of initial aqueous phase), and a second centrifugation was carried out to separate PT and the ethyl butyrate phase. However, as it was previously described in Section 3.1, these reaction conditions produced higher PC conversion and consequently, higher choline concentration than that achieved in the absence of $CaCl_2$. Hence, in the presence of $CaCl_2$, PT production in the second trials was almost negligible in both reactions studied (A and B).

3.3. *Recovery of phosphatidyl-tyrosol and tyrosol from reaction mixtures*

The same reaction conditions (A, B, C) utilized in the Section 2.5 were used in this section to obtain a purified PT. Table 5 shows the compositions and yield of the final dried and grinded product attained. It should be mentioned that after the centrifugation step in these three reaction mixtures, a triphasic system comprised of an aqueous phase, ethyl butyrate, and PT produced was observed. On the other hand, the ethyl butyrate phase, containing mainly tyrosol, was easily recovered (above 90% (w/w) of initial ethyl butyrate. Then several centrifugation stages were effected to separate aqueous phase and PT. It should be noted that this biphasic system trapped a low amount of ethyl butyrate which produced partial contamination of PT with tyrosol. Finally HPLC analysis showed that isolation of PT from the reaction mixture (C) with

Table 5 – Recovery of phosphatidyl-tyrosol from reaction mixtures.

Reaction name	$CaCl_2$ concentration (mmol/L)	Isolated powder (mg)	PT composition % (w/w)	PC composition % (w/w)	Remaining Tyrosol composition % (w/w)	Squalene composition % (w/w)	PT isolation yield % (w/w)
A	0	3130	69	20	9	1	54
B	0	1293	65	16	16	3	47
C	0	215	84	9	7	0	40
B	67	1156	97	0	2	1	70

the lowest concentration of PC assayed (20.8 mmol/L), yielded a final product with 84% by weight of PT, moreover PT isolation yield was 54%. The isolation procedure of the reaction mixture (B) with 80.3 mmol/L of PC produced a final product with 61% by weight of PT, and a PT isolation yield of 47%. However, at the highest substrates concentration of PC studied (reaction A) very difficult separation between phases was observed in the centrifuge tubes and finally a product containing 65% (w/w) of PT was achieved. At these conditions PT isolation yield was only 40%.

Then the influence of $CaCl_2$ on the PT isolation was also evaluated. It should be pointed out that reaction mixture (C) was not included here because the objective of this section was isolaton of PT from reaction mixtures containing higher concentrations of PC. Thus, $CaCl_2$ (67 mmol/L) was added to the reaction A and B to increase ionic strength in the aqueous phase and change the partition of PT between both phases. At these reaction conditions, the aqueous phase was easily separated after centrifugation (above 90% of initial aqueous phase), and a second centrifugation was carried out to separate PT and the ethyl butyrate phase. It should be pointed out, as it has been described in Section 3.1, that 67 mmol/L of $CaCl_2$ produces even higher PC conversions. By this procedure easier isolation of PT and lower tyrosol content in the final product was achieved in B reaction. The yield of the PT isolation was 70%, and a final product with 97% of PT was produced. But, similarly to that observed in absence of $CaCl_2$, at the highest substrate concentration of PC studied (reaction A) in presence of $CaCl_2$ (67 mmol/L), incomplete phase separation was attained.

A preliminary study to find out and compare salt content between initial PC and produced PT was also effected by determining the weight percentage of ashes in the different products under study. Hence, ash content of phosphatidylcholine was 3% (w/w), and the ash content of the PT purified was 15%, 7%, 4% and 11% in A, B, C, and B with 67 mmol/L $CaCl_2$ respectively. In addition, purified PT contained ca. 4% of water (w/w).

3.4. *Scale-up of the transphosphatidylation reaction*

In order to obtain a highly purified PT, the two main stages involved in the procedure, namely transphosphatidylation and purification, were scaled-up. First, 660 g of reaction mixture were prepared and then a continuous centrifuge was used to separate the product mixture and collect PT inside of a clarifying cylinder. Fig. 4 shows a diagram of the PT production and semi-continuous separation system. It should be indicated that the clarifying cylinder volume is 250 mL. During the centrifugation this volume was comprised of the aqueous phase and PT. It was also observed that volumes of aqueous phase and PT higher than 250 mL partially dragged out PT by the upper outlet simultaneously with the ethyl butyrate phase. For this reason reaction chosen to scale-up to $660 \times g$ was 417 mmol/L of tyrosol, 83.3 mmol/L of PC, 30 mmol/L of squalene, 211 mL of aqueous phase, and 422 mL of ethyl butyrate with 40 mg of PLD (1500 units/mg) since this concentration produces a mixture of aqueous phase and PT of ca 250 mL. On the contrary, 166.6 mmol/L of PC produced a volume of PT and aqueous phase superior to 250 mL, and for this reason, it was discarded.

At this reaction conditions the mixture was maintained 15 h and then it was delivered to the thermostatized continuous centrifuge. Most of the ethyl butyrate phase was removed by the upper outlet and the aqueous phase was collected via the discharge outlet. On the other hand, PT was collected inside the clarifying cylinder. The two liquid phases were mixed again and introduced in the centrifuge to recover residual PT as a solid phase into the clarifying cylinder. The two solid phases were mixed, dried and grinded. Finally 41 g of a powder with 94% PT composition and 6% of tyrosol were produced. The yield of the PT isolation procedure was 86%, the ash content 10% (w/w), and the humidity ca. 4% of water (w/w).

4. Conclusions

The present study shows a GRAS biphasic system to carry out the transphosphatidylation of PC with tyrosol catalyzed by a food grade PLD from *Actinamadure* sp. The reaction medium comprised of an aqueous phase and ethyl butyrate has been considered as an alternative to other commonly used biphasic systems utilizing more harmful organic solvents. Likewise it has been also showed that PT can be produced and purified from this reaction medium to obtain a new food ingredient.

In particular, several reaction parameters as substrate ratios, substrate concentrations, enzyme loading, aqueous phase composition and enzyme reuse to achieve an efficient transphosphatidylation reaction have been studied. By this methodology reactions with PC 83 and 166 mmol/L produced high concentrations of phosphatidyl-tyrosol (81 and 157 mmol/L) and PC conversion (ca. 97% and 94% (w/w)) using low enzyme loading (1% (w/w)).

Moreover, isolation of the new phospholipid formed was improved by the addition of 67 mmol/L of $CaCl_2$ to the reaction mixture and the subsequent centrifugation at 40 °C allowed a highly purified phosphatidyl-tyrosol (97% (w/w)).

Furthermore, the process has been scaled up to achieve 40 g of phosphtidyltyrosol by a simple methodology comprised of a 1 L stainless steel reactor coupled to a thermostatized continuous centrifuge. This procedure is able to produce high amounts of phosphatidyl-tyrosol and other similar modified PLs that can be utilized in cosmetics, foods and health applications.

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Novel and efficient solid to solid transphosphatidylation of two phenylalkanols in a biphasic GRAS medium

Victor Casado[a], Guillermo Reglero[a,b], Carlos F. Torres[a,*]

[a] Departamento de producción y caracterización de nuevos alimentos, Instituto de Investigación en Ciencias de la Alimentación (CSIC–UAM), C/ Nicolás Cabrera 9, Universidad Autónoma de Madrid, 28049 Cantoblanco, Madrid, Spain
[b] IMDEA-Food Institute, CEI (UAM-CSIC), C/ Faraday 7, 28049 Madrid, Spain

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ABSTRACT

A solid to solid reaction system for transphosphatidylation of phosphatidylcholine with two different phenylalkanols, namely tyrosol and hydroxytyrosol has been developed. The enzymatic reactions were carried out in the presence of a food grade phospholipase D at very mild reaction conditions (40 °C). All enzymatic reactions were performed in a biphasic GRAS medium comprised of sodium acetate buffer and ethyl butyrate. High volumetric productivity (up to 130 g of phosphatidylcholine per liter of reaction mixture) and equimolar ratio of substrates have been utilized. The ratio weight of product to weight of biocatalyst (100:1 w/w), has been also taken into account. At these reaction conditions, up to 150 mmol/L of both, phosphatidyltyrosol and phosphatidylhydroxytyrosol were obtained.

1. Introduction

Transphosphatidylation reactions are usually carried out biphasic systems comprised of a water-immiscible organic solvent (e.g., diethyl ether, chloroform, ethyl acetate) surrounding PC and an aqueous phase containing the enzyme and different nucleophiles [1]. However, utilization of toxic organic solvents for human consumption should be avoided. In this sense, synthesis of Phosphatidylserine in purely aqueous system has been developed [2]. In fact, the reaction system could be considered as a colloidal suspension. However, a serious drawback of this system is that it contains large quantities of water, which promotes the undesirable enzymatic hydrolysis of phosphatidylcholine. Besides the mentioned hydrolysis of the substrate, it also gives rise to the accumulation of considerable amounts of a secondary byproduct, phosphatidic acid (PA).

The benefits of using biosynthesis and also minimization of undesired byproduct formation can be further improved with a rational selection of the reaction medium [3]. In this sense, an ideal choice would be to carry out the enzyme-catalyzed synthesis of new phospholipids in a green, non-toxic and non-aqueous system. However, industrial application of transphosphatidylation reaction for the synthesis of new phospholipids is usually limited by its low productivity, as we need a medium where a polar reagent (nucleophile) and a phospholipid are able to react in presence of the biocatalyst. Moreover, for a bioprocess to be economically viable, certain process metrics must be achieved [4]. In the case of a biocatalytic process, two are particularly important. The usual requirement is to achieve product concentrations comparable to chemical processes of at least 50–100 g/L. Considering that in nature, enzymes work at millimolar levels of substrate, it is necessary to make them work away from their natural conditions. Process development as well as protein engineering solutions, are available to achieve such concentrations while maintaining sufficient activity. The other metric is dependent on the cost of the enzyme and is best expressed as the gram product/gram or activity unit of biocatalyst. For commercial processes, this metric needs to be 1000 for an enzyme and 15 for a whole-cell system (reflecting the different costs of each). Techniques such as immobilization become important to provide the ability to recycle the catalyst (in particular if it is an enzyme).

In order to achieve sufficient productivity, a plausible strategy concerns the removal of the reaction product. Many biocatalytic reactions, which are converting non-natural substrates at high concentration, become limited by the product (which can degrade, be inhibitory or toxic to the biocatalyst). The use of in situ product removal (ISPR) where the product is removed during the process can therefore lead to far higher productivity. ISPR methods can increase the productivity or yield of a given biocatalytic reaction by any of the following means [5]: overcoming inhibitory or toxic

* Corresponding author. Tel.: +34 910017912.
E-mail address: Carlos.torres@uam.es (C.F. Torres).

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effects [6]; shifting unfavorable reaction equilibrium [7]; minimizing product losses owing to degradation or uncontrolled release; and [8] reducing the total number of downstream-processing steps. The second strategy concerns the control of the supply of substrate. The use of an auxiliary phase (solid resin or organic solvent) to remove the product can be extended to incorporate additional supplies of the substrate. In many situations, the substrate is inhibitory, harmful, or insoluble at the concentration required, and methods using feeding or an auxiliary phase are required to overcome this. For example, two-phase systems can lead to considerable productivity gains, although the added complexity must be offset against the benefits [9]. Of particular concern is the use of organic solvents (with requirements for flameproof facilities) for which the use of newer options such as ionic liquids [10,11] or green solvents might, in some cases, provide a suitable alternative [12].

Finally, keeping all these goals in mind, a reaction medium selection for industrial application of transphosphatidylation reactions for solid-to-solid synthesis in green solvents should be accomplished. To our knowledge, this strategy has been related to the synthesis of protected peptides, beta-lactam antibiotics, glycosides, glycamides and esters starting from suspended substrates [13] but it has not been previously reported for the preparation of new phospholipids. In our previous study we have developed a suitable biphasic system to carry out the trasphosphatidylation between phosphatidylcholine and tyrosol [14]. Consequently, the present study shows a novel approach to improve and optimize the reaction conditions of the transphosphatidylation reaction between phosphatidylcholine and two nucleophiles namely, tyrosol and hydroxytyrosol, in terms of stoichiometry, substrate concentration, and biphasic medium composition intended to be used for scaled-up and industrial applications. In addition, a very simple downstream process for product recovery has been also described.

2. Materials and methods

2.1. Materials

Hydrogenated phosphatidylcholine, 90% (PC), was purchased from lipoid (Cham, Switzerland). Powdered preparation of *Actinamadure* Sp phospholipase D (EC 3.1.4.4) was acquired from Meito Sangyo CO (Nikko-Cho, Fuchu, Tokyo, Japan). According to the vendor specifications PLD contained 1500 U/mg (1 PLD Unit is defined as the amount of enzyme producing 1 μmol/h of choline from L-a-phosphatidylcholine when the enzyme solution is reacted at pH 8 at 30 °C). Tyrosol (98.0% w/w purity) was purchased from TCI Europe (Zwijndrecht, Belgium). Hydroxytyrosol was acquired from SEPROX (Madrid, Spain). Anhydrous calcium chloride (PRS Grade), formic acid (98%), ethyl butyrate (99.5%), squalene (97%) triethylamine (99.5%), phosphatidic acid (1,2-dioleyl-sn-glycero-3-phosphoric acid monosodium salt, 99%), and sodium acetate trihydrate (99%) were acquired from Sigma–Aldrich (St. Louis, MO, USA). Chloroform, 2-propanol, hexane, acetic acid, and methanol (HPLC grade) were purchased from Lab-Scan (Dublin, Ireland).

2.2. Phosphatidylcholine transphosphatidylation

Different quantities of PC and phenylalkanols (tyrosol or hydroxytyrosol) were added to a mixture of ethyl butyrate and sodium acetate buffer in a 60-mL flask and mixed by swirling. The total volume of the reaction mixtures was 2.4 mL. The aqueous phase was comprised of 0.2 M sodium acetate buffer (pH 5.6). 1% (w/w) of squalene (regarding total reaction mixture) as internal standard was dissolved into the ethyl butyrate phase. Then, phospholipase D (1% w/w) was added to the mixture. The flasks were placed in a temperature-controlled orbital shaker (IKA KS 4000, Staufen, Germany) at 40 °C and 200 rpm.

Additionally, to improve the rate of solubilization of solid reactants in the reaction mixture, commercial tyrosol was grounded in Retsch mill (Grindomix, Haan, Germany) and sieved through different sieves (from 500 to 100 μm). To minimize the influence of particle size on reaction rate, the transphosphatidylation took place with tyrosol below 100 μm particle size. The other reactant, hydrogenated phosphatidylcholine, was not grinded because of its original average particle size was below 100 μm. In order to make results readily comparable with each other, all concentrations have been expressed as mmols per total volume of both organic and aqueous phase (free of hydration and mineral salts).

The reaction mixture was stopped after different time intervals and extracted with an appropriate volume of chloroform/methanol/water/phosphoric acid (8/4/2/2, v/v/v/v) for analytical purposes. Centrifugation separated the mixture into two phases, an upper aqueous phase and a lower phase. The lower phase was recovered and analyzed by HPLC.

In order to determine the experimental error, some replicates of the mentioned procedure were carried out. In all reactions using 83.3 mmol/L PC, the standard deviation for concentration of phosphatidyltyrosol (PT) and phosphatidylhydroxytyrosol (PHT) produced, remaining PC, remaining tyrosol, and remaining hydroxytyrosol was <2 mmol/L. Regarding PC conversion, the standard deviation was lower than 2%.

In all reactions using 167 mmol/L PC, the standard deviation for concentration of PT and PHT produced, remaining PC, remaining tyrosol, and remaining hydroxytyrosol was <6 mmol/L. Regarding PC conversion the standard deviation was lower than 3%.

In addition, transphosphtidylation reactions utilizing 167 mmol/L of both reagents (PC and phenylalkanol) in a biphasic medium comprised of ethyl butyrate and sodium acetate buffer, 2/1 and 1/1 (v/v), for PT and PHT respectively, were carried out at 24 mL scale. These reactions were effected to purify PT and PHT via semipreparative HPLC and also to study the direct recovery of PT and PHT from the product mixtures. Flasks were placed in a temperature-controlled orbital shaker (IKA KS 4000, Staufen, Germany) at 40 °C and 200 rpm approximately 18 h.

2.3. HPLC analyses

1 μL of the final transparent solutions were analyzed on a Luna 5 μm HILIC diol column (250 mm, 4.60 mm, Phenomenex, Torrance, CA, USA) coupled to an agilent (Santa Clara, CA, USA) 1200 Series HPLC containing a temperature-controlled column compartment, quaternary pump, autosampler, vacuum degasser, and a dual detection system comprised of an evaporative light scattering and a diode array detector.

The flow rate was 1.5 mL/min. A splitter valve was used after the temperature-controlled column compartment and only 30% of the mobile phase was directed through the detector (3.5 bar and 41 °C). The column temperature was maintained at 55 °C. The mobile phase for the analysis of the product mixtures from PC transphosphatilydation with tyrosol consisted of a binary gradient of (A) hexane/2-propanol/acetic acid/triethylamine (815/170/15/0.8, v/v/v/v), (B) 2-propanol/water/acetic acid/triethylamine, (837/140/15/0.8, v/v/v/v). The method starts at 3% B increasing up to 15% in 20 min. The percentage of B was again reduced to 3% in 2 min and the initial conditions were maintained for 10 min.

The mobile phase for the analysis of the product mixtures from PC transphosphatilydation with hydroxytyrosol consisted of a ternary gradient of (A) hexane, (B) hexane/2-propanol/acetic acid/triethylamine (815/170/15/0.8, v/v/v/v), (C) 2-propanol/water/acetic acid/triethylamine, (837/140/15/0.8, v/v/v/v). The method starts at 50% of A and 50% of B for 5 min

increasing up to 100% of B in 5.1 min. Then up to 12% of C is added from 5 min to 7 min increasing up to 40% of C at 20 min. Then 100% of B is restored at 35.5 min and initial conditions at 36 min. These initial conditions are maintained for 9 min.

The mobile phase for the analysis of phosphatidic acid consisted of a binary gradient of A: chloroform/methanol/ammonium hydroxide, (80/19/0.5, v/v/v) B: chloroform/methanol/water/ammonium hydroxide, (60/34/5.5/0.5 v/v/v/v). The flow rate was 2 mL/min and the column temperature was maintained at 30 °C.

Identification and quantification was carried out by using standards for each lipid class involved in the transphosphatilydation reaction. In order to minimize error when using HPLC with ELSD, rigorous calibration curves for each lipid class were developed for each set of samples injected, since the detector response was non-linear and specific to each compound.

2.4. Purification of PT by semi-preparative HPLC coupled to a fraction collector

A modified version of the methodology for HPLC analysis was utilized to purify PT to be used as a standard for HPLC analyses. A reaction mixture comprised of 24 mL of biphasic medium was prepared to obtain enough material to purify PT via semi-preparative HPLC. The reaction mixture was stopped after 18 h and extracted with an appropriate volume of chloroform/methanol/water/phosphoric acid (8/4/2/2, v/v/v/v). Centrifugation separated the mixture into two phases, an upper aqueous phase and a lower phase. The lower phase was recovered and purified via semi-preparative HPLC. For this purpose, a fraction collector coupled to the HPLC was utilized. The flow rate was 6 mL/min. A splitter valve was used after the temperature-controlled column compartment and only 10% of the mobile phase was directed through the ELSD detector (3.5 bar and 41 °C). The column, Kromasil Sil column (5 μm, 250 mm, 10 mm), acquired from Análisis Vínicos (Tomelloso, Spain), was maintained in a temperature-controlled column compartment at 55 °C. The mobile phases utilized were the same as those described in HPLC analysis section of PC transphosphatilydation with tyrosol but with a different gradient elution. Briefly, the method started at 1% B increasing to 25% in 20 min. This percentage of B was maintained 5 min and was reduced to 1% in 2 min, and the initial conditions were maintained 10 min. Several consecutive injections of 200 μL of sample were effected to obtain enough PT to be used as analytical standard.

2.5. Purification of PHT by semi-preparative HPLC coupled to a fraction collector

Similarly to the purification of PT previously described (Section 2.4), a reaction mixture comprised of 24 mL of biphasic medium was prepared to purify PHT. The reaction mixture was stopped after 18 h and extracted with an appropriate volume of chloroform/methanol/water/phosphoric acid (8/4/2/2, v/v/v/v). Centrifugation separated the mixture into two phases, an upper aqueous phase and a lower phase. The lower phase was recovered and purified via semi-preparative HPLC. A fraction collector coupled to the HPLC was also utilized. The flow rate was 5 mL/min. A splitter valve was used after the temperature-controlled column compartment and only 10% of the mobile phase was directed through the detector (3.5 bar and 41 °C). The column Kromasil Sil column (5 μm, 250 mm, 10 mm), acquired from Analisis Vinicos (Tomelloso, Spain), was maintained in a temperature-controlled column compartment at 55 °C. The mobile phase utilized consisted of a binary gradient of A: hexane and B: hexane/2-propanol/acetic acid/triethylamine (815/170/15/0.8, v/v/v/v). Briefly, the method started at 1% B increasing to 13% in 10 min, and then increasing to 50% in 1 min. This percentage of B was maintained 16 min and reduced to 1% in 1 min, and the initial conditions were maintained 10 min. Several consecutive injections of 200 μL were effected to obtain enough PHT to be used as analytical standard.

2.6. Recovery of phosphatidyl-tyrosol and phosphatidylhydroxytyrosol from reaction mixtures

After 18 h, both transphosphatidylation reactions at 24 mL scale were removed from the orbital shaker. First, the upper phase was separated by centrifugation at 8960 × g. Then a second centrifugation with the interface and the lower phase was performed to obtain a solid residue after removing the lower phase. The solid residue was then washed with ethyl butyrate (10 mL) and centrifuged at 8960 × g. The new solid residue attained was washed again with water (10 mL) and centrifuged at 8960 × g. The final solid residue was evaporated in a rotary evaporator and PT and PHT were obtained as solid products, and grounded in a Retsch grindomix mill. Finally, dried PT and PHT residues attained were analyzed by HPLC, and PT and PHT compositions, including PC and phenylalkanol remaining percentage of the mentioned dried products were determined.

2.7. Scaled-up of the transphosphatidylation reaction

Both PT and PHT transphosphatidylation reactions were scaled-up to 204 mL of biphasic medium in a 1 L stainless steel reactor coupled to a paddle stirrer at 200 rpm (Kiloclave, Buchi Glass Uster, Switzerland). The concentration of both substrates was 167 mmol/L, and the ratio of ethyl butyrate/sodium acetate buffer phase was 2/1 and 1/1 in PT and PHT transphosphatidylation, respectively. Each reaction mixture was thermostatized at 40 °C with a heating jacket coupled to the reaction vessel. After 18 h, the reaction mixtures were removed from the reactor and introduced in 250 mL centrifuge tubes and centrifuged at 8960 × g, at 40 °C for 10 min. After centrifuging, two liquid phases and a solid interphase were obtained from each product mixture. The solid interphases, were then washed with ethyl butyrate (100 mL) and water (100 mL) and centrifuged again. The solid residues attained after removing ethyl butyrate and water were then evaporated in a freeze-dryer and a dried PT and PHT residues were obtained, which were then grounded in the Retsch grindomix mill.

2.8. Determination of water content and ash content

A Karl Fischer automatic titrator 870 Titrino Plus (Metrohm, Herisau, Switzerland) with hydranal composite 5 (Sigma–Aldrich, St. Louis, MO, USA) was used to determine moisture. Ash content of the phosphatidyltyrosol and phosphatidylhydroxytyrosol produced was determined gravimetrically by using a muffle furnace.

2.9. Determination of PC, PT and PHT solubility

1 mL of supersaturated solutions of PC, PT, and PHT in ethyl butyrate were prepared in 4-mL flasks and mixed by swirling. The vials were kept 1 h at 40 °C. Then, 200 μL of the ethyl butyrate solutions were taken and the solvents were completely evaporated under nitrogen. The residues were utilized to calculate the concentration of PC, PT, and PHT soluble in ethyl butyrate.

3. Results and discussion

The application of enzyme-based catalysts is moving in the direction to improve yields and to allow the production of more-complex molecules. Similarly, bioreactor operation is moving from dilute to high concentrations of reactant and product in order

Table 1
Influence of the molar ratio in the transphosphatidylation between PC and two different phenylalkanols (Tyr, tyrosol, HT, hydroxytyrosol) after 24 h.

Phenylalkanol:PC ratio	Phenylalkanol (mmol/L)	PC (mmol/L)	Produced phospholipid (mmol/L)		Remaining PC (mmol/L)		Remaining phenylalkanol (mmol/L)	
			Tyr	HT	Tyr	HT	Tyr	HT
2:1	333	167	132	–	33	–	200	–
2:1	208	104	83		20		125	
2:1	167	83	64	–	19	–	102	–
3:1	208	62	51		11		157	
5:1	208	42	38		4		170	
10:1	208	21	19		1		188	
15:1	208	14	13		1		195	
2:1	208	104		83		21		125
3:1	208	62		52		11		156
5:1	208	42		38		4		171
10:1	208	21		19		1		189
15:1	208	14		13		1		196

to meet the volumetric productivities required in the industrial context. Although many biocatalytic processes are now finding applications in industry, there remain three fundamental barriers to their widespread use: (1) cheap and stable enzymes, (2) systematic design methods are needed to speed up the development of whole process sequences and to enable rational choices to be made between chemical and biocatalytic routes to the same end product, and (3) the low productivities that are often observed with biocatalytic systems, relative to the chemical equivalent, must be enhanced in order to develop more-economic processes. In this sense, in a preliminary study of the transphosphatidylation reaction between PC and tyrosol and hydroxytyrosol, the concentration of PC was increased from 21 to 333 mmol/L with a molar ratio 1:10 of PC:phenylalkanol. Neither PT nor PHT were observed when 333 mmol/L of PC was utilized. The highest concentration that produced positive results was 167 mmol/L and 83 mmol/L for tyrosol and hydroxytyrosol respectively. The enzyme loading in these transphosphatidylation reactions was 1% w/w of the reactants, which represents a ratio, grams of reactants to grams of biocatalyst of 100 to 1.

Next, different molar ratios of phenylalkanols to PC were studied. The results are shown in Table 1. It can be observed that similar PC conversion was attained with both tyrosol and hydroxytyrosol. It should be also noted that regardless the molar excess of phenylalkanol utilized, similar PC conversion was observed except for molar ratios of phenylalkanol to PC of 3 to 1 and 2 to 1, which could indicate that the rate limiting substrate in the transphosphatidylation reaction is PC. In addition, 3 different concentrations of tyrosol (from 167 to 333 mmol/L) and PC (from 83 to 167 mmol/L) were utilized at a molar ratio of phenylalkanol to PC of 2–1. In order to reduce the utilization of the expensive HT, two of the mentioned reactions (row 1 and 3 of Table 1) were studied only with tyrosol. PC conversion was ca 80% in these three cases studied. The enzyme loading in all these transphosphatidylation reactions was 1% w/w of the reactants. However, in all these reactions a huge concentration of phenylalkanols remains at the end of the bioprocess which complicates downstream processing for the recovery of the new phospholipid produced.

For this reason, the transphosphatidylation was carried out also at equimolar concentration of both substrates at two different concentrations, namely 83 and 167 mmol/L. The results (Table 2) indicate that lower reaction conversions were attained at these stoichiometric conditions regardless the concentration of substrates utilized. The enzyme loading in these transphosphatidylation reactions was 1% w/w of the reactants. PC conversions of ca 60% and 35% were obtained using substrate concentrations of 83 and 167 mmol/L, respectively. There was found to be a correlation between changes in the initial substrate ratio and the pH of the liquid phase, and it was demonstrated that even a slightly unequal substrate ratio can have a large effect on rate [15]. However, unequal substrates ratio means that at the equilibrium an excess of one unreacted substrate will be present, so other methods are generally preferred. Following these considerations, the pH of these systems can be controlled by the addition of inorganic salts (acids or bases). On the basis of these results it is possible to create a model for the prediction of the initial pH and the change of pH during product formation to explain both final yields and the enzyme kinetics in this system [16]. In such systems salts can be used also when thermodynamics are unfavorable and precipitation is not achieved. The addition of specific counter-ions that form poorly soluble salts with an ionic form of the product of a reaction (and not with the substrates) lead to product salts precipitation from the reaction mixture. There are a number of reported 'solid-to-solid' reactions where products precipitated as salts rather than

Table 2
Transphosphatidylation reaction between PC and two different phenylalkanols (Tyr, tyrosol; HT, hydroxytyrosol) at equimolar concentration of substrates and in the presence of $CaCl_2$.

$CaCl_2$ (mmol/L)	Phenylalkanol (mmol/L)	PC (mmol/L)	Produced phospholipid (mmol/L)		Remaining PC (mmol/L)		Remaining phenylalkanol (mmol/L)	
			Tyr	HT	Tyr	HT	Tyr	HT
0	83	83	47		36		36	
67	83	83	70		12		12	
0	167	167	55		110		110	
67	167	167	145		21		21	
0	83	83		45		38		38
67	83	83		71		12		12
0	167	167		58		125		125
67	167	167		137		30		30

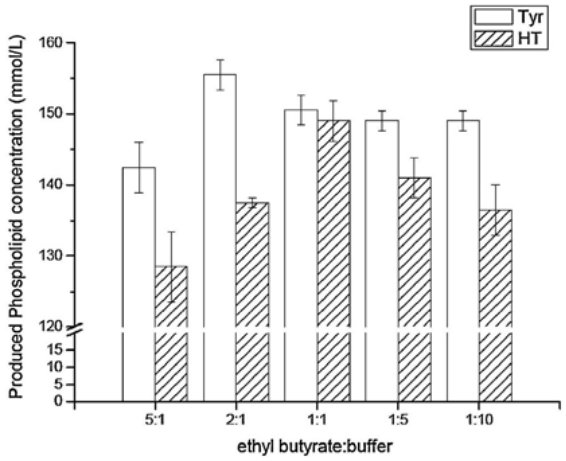

Fig. 1. Influence of the relative volumetric proportion of ethyl butyrate to buffer in the equimolar transphosphatidylation reaction between PC and two different phenylalkanols. Tyr, tyrosol; HT, hydroxytyrosol.

as neutral compounds. Taking these facts into consideration, $CaCl_2$ was added to the reaction mixtures. Table 2 also shows that addition of $CaCl_2$ to the equimolar reaction mixtures increased the PC conversion up to c.a. 85%. Further experiments were carried out with 67 mM of $CaCl_2$.

Next, the relative volumetric proportion of aqueous phase and ethyl butyrate was investigated. It has been pointed out that the thermodynamic feasibility of 'solid-to-solid' reactions is solvent independent [17] at fixed water activity. This means (theoretically) that if product precipitation is favored in aqueous substrate suspension it should also be feasible in other solvents. It should be also pointed out that in these precipitation-driven reactions, the product accumulates in both, solid and liquid phases of the reaction mixture. Therefore, two different yields can be distinguished. These are the solid yield (the yield of precipitated product only), and the total yield (the product yield in both the solid and the liquid phases). These yields vary depending on the solvent in which the reaction is carried out. As a rule of thumb it has been suggested that rather hydrophilic solvents (low log*P*) give the best results for both peptides and sugar fatty acid esters [18]. An inverse correlation between product yield and product solubility was suggested to explain these observations [19]. In order to study the influence of the relative volumetric proportion of aqueous face and ethyl butyrate, the concentration of both substrates chosen was 167 mmol/L. The reactants/enzyme ratio in these transphosphatidylation reactions was 100/1. The results in Fig. 1 indicate that different optimal volumetric ratios of ethyl butyrate to buffer were obtained for tyrosol (2 to 1) and hydroxytyrosol (1 to 1). Moreover, in all cases studied, reaction conversion was lower for hydroxytyrosol than that for tyrosol. These differences observed could be related with the different solubility of tyrosol and hydroxytyrosol in ethyl butyrate and buffer. Less polar tyrosol (Log*P* 1.05 ± 0.34) [20] may be mainly solubilized in ethyl butyrate and is not affected by the buffer proportion in the reaction mixture when the proportion of ethyl butyrate to buffer is lower than 2. On the contrary, more polar hydroxytyrosol (Log*P* 0.55 ± 0.65) could be partitioned in both ethyl butyrate and buffer. For this reason, increasing proportions of buffer above 1:1 dilute the effective concentration of HT and lower transphosphatidylation reaction is observed. On the contrary higher proportions of buffer in the case of tyrosol had negligible effect on the transphosphatidylation reaction. In addition solubilities of PC, PT, and PHT in ethyl butyrate were determined.

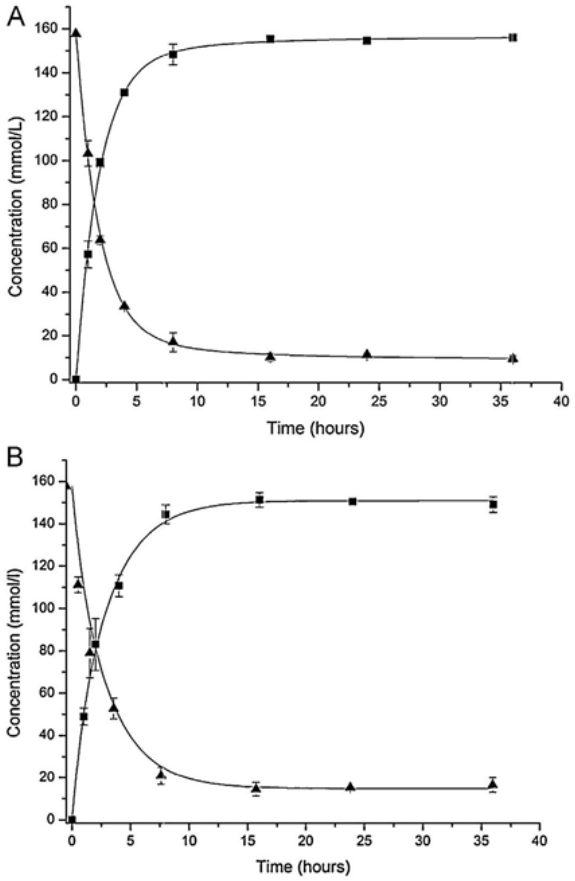

Fig. 2. Time course of the transphosphatidylation reaction between PC and tyrosol (A) and hydroxytyrosol (B) at equimolar conditions.

Only 2.00 ± 0.87, 10.20 ± 0.47, and 3.67 ± 0.01 mmol/L of PC, PT, and PHT were solubilized in ethyl butyrate. This result indicates that transphosphatidylation occurs in a solid to solid reaction system. It could be pointed out that the rate of solubilization of PC in the reaction mixture is the rate limiting step in the transphosphatidylation reaction. Besides, the reaction product accumulates as a solid insoluble in the reaction mixture which shifts the reaction equilibrium toward the formation of product. This fact improves PC conversion and also permits one to increase volumetric productivity of the reaction system by using PC concentration up to 167 mM.

The undesirable enzymatic hydrolysis of phosphatidylcholine associated with the transphosphatidylation reaction was also considered by measuring the accumulation of the phosphatidic acid in the product mixtures. This undesirable byproduct was not detected in any of the transphosphatidylation reactions investigated which indicates null hydrolysis associated with the transphosphatidylation reaction.

Finally, the time course of both transphosphatidylation reactions was studied (Fig. 2). These reactions were carried out at 167 mmol/L of both substrates and a ratio of ethyl butyrate to buffer of 2–1 and 1–1 for tyrosol and hydroxytyrosol respectively. The enzyme loading in these transphosphatidylation reactions was 1% w/w. From Fig. 2, it can be observed lower reaction rates for hydroxytyrosol than that for tyrosol. It has been previously described that the meta-hydroxyl group in the benzene ring could slightly inhibit

the transphosphatidylation by PLD [21]. In both cases the reaction equilibrium was reached in approximately 15 h with PC conversion higher than 90% in both cases under study.

3.1. Recovery of the phosphatidyltyrosol and phosphatidylhydroxytyrosol produced

Transphosphtidylation with 167 mmol/L of both PC and each phenylalkanol, in 24 mL of biphasic medium (comprised of ethyl butyrate and sodium acetate buffer 2/1, and 1/1 (v/v) for PT and PHT respectively) were prepared to recover PT and PHT. It should be mentioned that after the centrifugation step in the reaction mixtures, a triphasic system comprised of an aqueous phase, ethyl butyrate, and a solid phase containing mainly of PT or PHT produced was observed. Then, two centrifugation stages were performed: firstly to separate aqueous phase (above 90% of initial aqueous phase was retired), and then to separate ethyl butyrate phase and the solid phase. It should be noted that a low amount of ethyl butyrate was trapped within the solid phase which produced partial contamination of PT and PHT with unreacted tyrosol and hydroxytyrosol, respectively. Then the solid phase obtained, was washed with 10 mL of ethyl butyrate and 10 mL of water. By this procedure PT and PHT with lower tyrosol and hydroxytyrosol content were achieved. Two final solid products with 94% of PT (6% tyrosol and 0% PC) and 91% of PHT (6% HT Y 3% PC) were attained. The ash content of both products was 10% (w/w), and the water content ca. 4% and 5% (w/w) in PT and PHT respectively.

3.2. Scaled-up of the transphosphatidylation reaction

Finally, the process was scaled-up to a 204 mL of reaction mixture in a 1 L stirred tank reactor to test the reproducibility of the procedure. Similarly to the reaction in 24 mL of biphasic medium, the product mixture was centrifuged twice to remove buffer and ethyl butyrate phases and then washed with ethyl butyrate and distillate water to attain PT and PHT with lower content of tyrosol and hydroxytyrosol. Finally 26 and 24 g of PT and PHT powder were attained. The product PT was comprised of 88% PT, 7% tyrosol, and 5% PC (w/w). Similarly, the product PHT was comprised of 86% PHT, 8% HT and 6% of PC (w/w). The ash content was ca.10% (w/w) in both cases, and the humidity was c.a. 5% and 3% (w/w) for PT and PHT respectively.

The product yield, defined as weight percentage of pure product attained, at 24 mL scale, was 86 and 81% for PT and PHT respectively. Similarly, product yield of 85 and 76% for PT and PHT respectively, was attained at 204 mL. These results indicate that the procedure described is easily scalable with comparable results although some improvements in the washing and centrifugation steps could be further investigated.

A slightly lower purity of PT and PHT was obtained in the 204 mL biphasic medium compared to that at 24 mL. This result could be related with the different reactors geometry and shaking devices utilized in both procedures. Besides, more efficient removal of the remaining PC at 24 mL scale than that at 204 mL scale by the washings, could also improve slightly the purity attained at 24 mL scale. On the contrary, similar content of unreacted phenylalkanols were obtained in both procedures. These results suggest that both reaction conversion and washing procedures should be carefully considered at 204 mL scale in order to improve purity and remove more efficiently unreacted PC. However, it should be taken into account that more efficient washing procedure could also lead to lower yields of PT and PHT by partial solubilization of the reaction products through the washings.

Finally, the water content of the four products obtained was similar which indicates that rotor evaporator and lyophilization were adequate to remove humidity from the final products at both scales investigated.

4. Conclusions

The present study shows a very efficient solid to solid transphosphatidylation reaction in a GRAS biphasic reaction medium that can be easily scaled up for the production of highly valuable new phospholipid at pilot plant facilities. Besides, phosphatidic acid was not produced in the transphosphatidylation reactions which could indicate that the low solubility of PC, PT, and PHT in the reaction medium could preserve them from the undesirable hydrolysis reaction. It should be also noted that each nucleophile utilized in transphosphatidylation reactions may require specific reaction conditions in term of salts, organic phase, and aqueous phase compositions and proportions, in order to achieve optimum conversion and product purity. Taking into account that the enzymatic reaction is carried out at equimolar conditions of both reactants, a very simple downstream processing is required to recover highly purified products. This procedure shows an adequate tool to prepare enough amounts of these two new phospholipids to test their potential as technological antioxidants and also to further investigate their potential biological activities.

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DISCUSIÓN GENERAL

7.2 DISCUSIÓN GENERAL

7.2.1 Antecedentes

La producción de un fosfolípido (PL) estructurado requiere el estudio de diversas técnicas y metodologías que aseguren una correcta reacción de síntesis, monitorización, extracción y análisis del compuesto obtenido. El trabajo "Lipids as Delivery Systems to Improve the Biological Activity of Bioactive Ingredients" se ha desarrollado como base en la elección de la metodología para la producción de un lípido portador. El artículo muestra el estado del arte de la biocatálisis enzimática en el campo de los lípidos portadores, por tanto, sirve como punto de partida para valorar el potencial uso de diferentes compuestos, tales como, triglicéridos (TGs), diglicéridos (DGs), ácidos grasos, alcoholes grasos, alquilgliceroles, esteroles y PLs. Éstos han sido estudiados como lípidos portadores de diferentes compuestos bioactivos y se han observado grandes diferencias en los métodos empleados, así como en los resultados obtenidos según sea el lípido utilizado.

Tras estudiar las diversas posibilidades observadas, se planteó la utilización de PLs y compuestos fenólicos para conseguir ingredientes funcionales. Los PLs fueron elegidos por su diferenciación frente a TGs, DGs, esteroles, ácido grasos y alcoholes grasos, ya que pueden incorporar compuestos bioactivos en su cabeza polar, mediante el uso de fosfolipasas. La posibilidad de incorporar compuestos bioactivos en la cabeza polar de un fosfolípido permite modificar diversos alcoholes con marcada actividad biológica. Además el carácter anfifílico de los PLs, proporciona una funcionalidad química muy diferente a los derivados lipídicos producidos de esta manera. Por otro lado, un aspecto de interés adicional en el desarrollo de lípidos portadores, es que puede existir actividad biológica mutua, tanto del vehículo portador utilizado como del compuesto incorporado en su cabeza polar. En éste grupo de lípidos portadores se encuentran los PLs, que por sí solos, podrían ser incorporados en alimentos como ingredientes funcionales. Por último, a diferencia de los lípidos neutros (LN), los PLs son los principales compuestos de la membrana celular y tras su modificación, podrían transportar estas moléculas funcionales a las membranas celulares de diferentes tejidos.

Se puede considerar una gran cantidad de posibles combinaciones entre PLs y compuestos bioactivos para formar lípidos portadores. La elección de la molécula bioactiva, se basa en su uso potencial como ingrediente funcional o nutracéutico, aunque, también en la vehiculización se tienen en cuenta otros aspectos de dicha molécula, como son: sus propiedades organolépticas, su solubilidad, bioaccesibilidad y biodisponibilidad y la estabilidad química, termolabilidad y susceptibilidad a la oxidación. Por todo ello se buscaron, compuestos de difícil aplicación directa en la formulación de alimentos funcionales o con limitada funcionalidad o biodisponibilidad. Tienen especial interés los ingredientes bioactivos que muestran propiedades antioxidantes con escasa solubilidad en determinadas matrices, y que tras su modificación tienen potencial en su aplicación como antioxidantes tecnológicos. Este es el caso de algunos compuestos fenólicos con actividad antioxidante, cuya biodisponibilidad y aplicabilidad en alimentos pueden ser mejoradas mediante el uso de lípidos portadores.

Es de resaltar, que se han desarrollado trabajos de síntesis de PLs estructurados con compuestos fenólicos, sin embargo, la mayoría de los procesos de obtención de PLs portadores, ya existentes en el ámbito científico, han sido desarrollados desde un punto de vista alejado a su posible aplicabilidad industrial y comercialización para el consumo humano. Disolventes tóxicos, enzimas no aptas para el consumo humano o baja productividad son usuales en la síntesis de estos nuevos fosfolípidos.

En el capítulo “Phospholipases in food industry : a review“ del libro “Lipases and Phospholipases; Methods and Protocols”, se pone de manifiesto la creciente utilización de fosfolipasas en diversos campos de la industria alimentaria. Este trabajo ha ilustrado con éxito la validez de los métodos utilizados en la industria y ha estudiado la viabilidad del uso de fosfolipasas en algunas de las aplicaciones más usuales en la actualidad. Se han analizado los ejemplos más representativos en la utilización de las fosfolipasas en la industria alimentaria como en aceites comestibles, productos lácteos, productos de panadería, agentes emulsionantes de huevo y soja, así como las tendencias actuales en el desarrollo de nuevas especies moleculares de PLs.

De esta forma, este capítulo permite valorar las diferentes posibilidades para sintetizar un fosfolípido portador. Entre los métodos estudiados se pueden observar diversas características en los procesos industriales de gran interés, tales como, su aplicabilidad

demostrada a gran escala, la utilización de fosfolipasas aptas para el consumo humano, la minimización en el uso de disolventes tóxicos, o el control de la productividad y la rentabilidad del proceso. Estas características son de gran relevancia para conseguir producir un fosfolípido estructurado que puede ser apto para el consumo humano de forma realista.

Por otro lado, se ha observado la diversidad de fosfolipasas microbianas, de mamíferos y de plantas que se están estudiando de forma continuada, así como, su disponibilidad comercial a escala industrial en la producción de alimentos. La PLD parece ser la fosfolipasa más indicada para la formación de un fosfolípido estructurado con un compuesto fenólico, ya que esta enzima tiene la capacidad de producir transfosforilaciones con alcoholes primarios y secundarios. Dos compuestos fenólicos con estas características y con demostrada actividad biológica son el tirosol y el hidroxitirosol (HT). Por tanto, se planteó la producción de dos PLs portadores con estos dos fenilalcanoles, dando lugar a fosfatidiltirosol (PT) y fosfatidilhidroxitirosol (PHT).

Se ha observado, que la aplicabilidad industrial de catalizadores enzimáticos pasa por la mejora del rendimiento en determinados procesos. Del mismo modo, muchos procesos requieren dar un paso cuantitativo en términos de concentraciones de reactivo y producto, con el fin de satisfacer las productividades volumétricas requeridas en el ámbito industrial. Aunque muchos de los procesos de biocatálisis se encuentran ahora aplicados en la industria, aún existen tres barreras fundamentales para su uso generalizado: la necesidad de enzimas baratas y estables, el desarrollo de métodos más sistemáticos y evitar las productividades volumétricas bajas, que a menudo se observan en los sistemas de biocatálisis, comparados con procesos puramente químicos. En este sentido, en el artículo “Production and Scale-up of phosphatidyl-tyrosol catalyzed by a food grade phospholipase D” se estudió la reacción de transfosfatidilación entre PC y el tirosol valorando el gasto enzimático, la simplicidad del proceso y la productividad. Por otro lado, se planteó en otro artículo, el estudio de la transfosfatidilación con HT y una posterior optimización del proceso.

En el artículo “Production and Scale-up of phosphatidyl-tyrosol catalyzed by a food grade phospholipase D” se describió la reacción de transfosfatidilación compuesta por

PC y tirosol, utilizando una PLD de *Actinamadure sp* (elegida por ser de grado alimentario). El medio de reacción estaba compuesto por un medio bifásico de etil butirato y tampón acetato-acético. Por lo general, los medios bifásicos son comúnmente utilizados en reacciones de transfosfatidilación, principalmente, el acetato de etilo o el dietil éter. Estos disolventes son utilizados en combinación con una fase acuosa tamponada[140, 180]. Sin embargo, el acetato de etilo y el éter dietílico son menos adecuados para aplicaciones en alimentos ya que poseen mayor riesgo por su toxicidad. Por lo tanto, para conseguir el medio bifásico adecuado, se estudiaron varias posibles fases lipídicas en base a su baja toxicidad y nula interferencia con la reacción y el análisis de HPLC. Ácido butírico, ácido oleico, butirato de etilo, triacetina, y tributirina fueron utilizados como fase orgánica para lograr una mezcla de reacción adecuada combinada con la fase acuosa. Finalmente, el sistema bifásico elegido fue tampón de acetato de sodio junto a butirato de etilo, ya que los estudios iniciales indicaron mayor conversión de PC a PT en este medio.

Es relevante indicar también que el medio de reacción influye posteriormente en el método de purificación del producto sintetizado. Por ello, se evaluaron los diferentes medios teniendo en cuenta la dificultad para extraer los nuevos PLs producidos de dichos medios.

Tras elegir el medio bifásico (tampón-butirato de etilo), fue necesario estudiar el curso de la reacción con el tiempo y la extracción cuantitativa de los PLs y fenoles de este medio bifásico con fines analíticos. La extracción de las mezclas de reacción con disolventes es necesaria para el seguimiento del bioproceso y su análisis mediante HPLC. Un problema encontrado en diferentes fases de este trabajo y muy característico en el campo de los PLs, es conseguir una extracción completa de mezclas compuestas por LN, PLs y sus derivados en determinados medios (como medios bifásicos). Esta complicación, también ha sido señalada por otros autores [158].

Durante la reacción de transfosfatidilación se producen cambios en el medio, esto dificultan la extracción y preparación de la muestra para analizar por HPLC y al tratarse de reacciones en medio bifásico, es difícil conseguir extraer completamente los PLs y los productos formados en la reacción. Por otro lado, al modificar parámetros de la reacción, tales como, concentración de sales o concentración de sustratos se puede influir en la eficacia del método de extracción. El método finalmente elegido para

conseguir la adecuada preparación de muestras consistió en una modificación del método Folch (extensamente utilizado en este campo) [162]. Esta variación se basó en la modificación de la proporción de los disolventes (cloroformo/metanol/agua (8/4/1.6, v/v/v)) y la aplicación de pequeñas cantidades de ácido fosfórico (hasta un 12% del total de disolventes). Con esta mezcla se consiguió extraer la totalidad de los compuestos de la fase lipídica. En este campo es necesario profundizar en el conocimiento de estas metodologías, ya que a día de hoy, existen pocos métodos sencillos y de amplia aplicabilidad, para extraer cuantitativamente diferentes mezclas de PLs.

Con el objetivo de conseguir una cuantificación lo más completa posible de las muestras, se desarrollaron varios métodos de cromatografía de líquidos. En primer lugar, dos nuevos métodos de HPLC con colector de fracciones fueron utilizados para la purificación de PT y PHT. Estos métodos fueron utilizados para obtener los dos patrones de PT y PHT necesarios en la monitorización de las reacciones posteriores. El equipo de HPLC acoplado a un colector de fracciones permitió obtener hasta 200 mgs de cada uno los nuevos PLs. Para obtener estas cantidades se realizó la reacción de transfosfatidilación en 24 ml de medio de reacción para obtener cantidades suficientes de productos de reacción. El mayor problema encontrado fue la presencia de ácidos y bases en las fases móviles utilizadas para la purificación de PT y PHT. Estos ácidos y bases contaminan el compuesto de interés tras la eliminación de la fase móvil (necesaria para obtener el patrón purificado). Además, PT y PHT quedaban en un medio altamente reactivo tras la posterior evaporación de la fase móvil. Esto obligó a eliminar de la fase móvil el ácido acético y la trietilamina utilizados, para evitar dicha contaminación. La purificación de estos PLs en ausencia de ácidos y bases en la fase móvil supuso una enorme dificultad debido a la perdida de resolución cromatográfica de estos compuestos, aunque finalmente lograron ser purificados.

Por otro lado, dos métodos de análisis de HPLC acoplados a un detector evaporativo de dispersión de luz (ELSD) fueron adaptados a las necesidades de este trabajo a partir de otro método previamente publicado [138]. Estos dos métodos diferentes fueron necesarios para la correcta elución de los dos derivados fosfolipídicos producidos (PT y PHT), debido a la diferente polaridad de tirosol e HT. La identificación y cuantificación fue llevada a cabo mediante la utilización de los patrones previamente purificados,

para obtener resultados con el mínimo error posible, y se realizaron rigurosas curvas de calibración para cada compuesto contenido en las muestras inyectadas. Esto es necesario ya que el detector ELSD tiene una respuesta no linear y específica para cada compuesto.

7.2.2 Producción de los fosfolípidos portadores

Los datos obtenidos en el estudio de la reacción de transfosfatidilación fueron indicados en concentraciones de sustratos o productos (en el medio de reacción), por ejemplo, 21 mmol/L de PC en 2,4 mL de medio bifásico. El objetivo de mostrar los resultados de esta manera es tener en cuenta la productividad del proceso de forma constante y poder comparar resultados a diferentes escalas (2,4 mL, 24 mL o escala planta piloto).

El estudio de la reacción de transfosfatidilación con tirosol, se basó en la experimentación en torno a cuatro variables; concentración de los sustratos, relación molar de sustratos, carga enzimática y concentración de cloruro cálcico.

Una primera transfosfatidilación fue ejecutada tomando como referencia una reacción previamente descrita en la bibliografía [149] en la que se usa etil acetato en el medio bifásico de reacción (relación tampón: etilacetato 1:2). En lugar de etil acetato, se usó el medio bifásico formado por tampón y etil butirato, la relación molar de PC: tirosol utilizada fue de 1:10, la concentración de PC fue de 21 mmol/L y se añadieron 1500 unidades de PLD/mg (1% de enzima, respecto a sustratos). Los resultados obtenidos fueron similares a los logrados con acetato de etilo como disolvente, ya que, la conversión de PC fue de 94 %.

Seguidamente, se estudió la concentración de los sustratos, se aumentó la concentración de los sustratos manteniendo constante el resto de variables de reacción. Se obtuvieron buenos resultados de conversión de PC (91 %) duplicando la concentración inicial mediante el uso de 42 mmol/L de PC. Sin embargo, concentraciones superiores produjeron conversiones de PC menores del 80%. En cuanto al tiempo necesario para la síntesis de PT, se observó que la reacción de transfosfatidilación a la concentración más baja de PC (21 mmol/L) requiere sólo 5 horas para obtener 19 mmol/L de PT, pero usando altas concentraciones de sustrato (reacción con 83 mmoles/L de PC) el tiempo aumento hasta las 15 h.

En relación a otros resultados publicados anteriormente, se ha descrito que la transfosfatidilación proporciona buenos resultados de conversión a bajas concentraciones de PLs [130]. En dicho artículo, se compara la transfosfatidilación a altas y bajas concentraciones de sustrato, un sistema bifásico compuesto por una disolución de PC en éter dietílico (106 mM) y una disolución saturada de serina (3,4 M) utilizando 1 g de dos preparaciones acuosas de PLD de *Streptomyces* dieron lugar a sólo el 36 % de PS. Sin embargo, una mezcla de reacción utilizando una disolución de PC en éter dietílico de 17,8 mM alcanzó una producción del 85 % de PS. Estos resultados se atribuyen a la inhibición de la PLD por las altas concentraciones de colina en el medio, procedente de la PC. Este estudio también indicó la dificultad en la eliminación de colina de la mezcla de productos al final de la reacción. Se ha descrito, que uno de los principales problemas asociados al escalar el proceso, es la inhibición del producto a altas concentraciones de sustrato y esto se traduce en bajos rendimientos. Como ya se ha mencionado, el problema está asociado con la concentración de colina que tiende a revertir el equilibrio de la reacción. Una posible solución es la adición de enzimas específicas de la colina como la colina oxidasa y catalasa, pero este doble sistema enzimático aumentaría drásticamente los costes del procedimiento, por lo que se descartó.

Seguidamente, se estudió la influencia de la carga enzimática en reacciones con diferentes concentraciones de sustratos. Se añadió PLD hasta llegar a 1 % ,5 % y 10 % respecto al peso de reactivos. La conversión de la PC utilizando 167 mmol/L de PC fue de 73%, 74% y 82%, usando 1 %, 5 %, y 10 % en peso de PLD. Por lo tanto, aunque la conversión de PC fue ligeramente más alta usando 5 % y 10 % de PLD, la mejora obtenida puede considerarse pequeña, y no compensa el incremento de coste del bioproceso que conlleva. Por ello, esta estrategia fue descartada.

Posteriormente se investigó la relación molar, diversas reacciones se llevaron a cabo modificando la concentración de los dos sustratos. De esta forma, relaciones molares entre PC y tirosol de 1:2, 1:3, 1:5, 1:10 y 1:15 fueron estudiadas. Los mejores resultados de conversión de PC (93 %) se consiguieron utilizando las concentraciones más bajas de PC (1:10 y 1:15), aunque obviamente, una alta concentración de tirosol remanente se encontró en el medio al parar las reacciones. Para obtener una alta conversión de PC junto a una alta concentración de PT, se estudió una reacción con

elevada concentración de PC y relación molar de 1:15. Efectivamente, la tasa de conversión de PC fue alta, pero el tirosol remanente en esta reacción se consideró excesivo. La relación molar 1:5 puede ser considerada como una proporción algo más adecuada para altas concentraciones de sustrato, ya que permite alcanzar un 90 % de conversión de PC y da lugar a una menor concentración de tirosol en el producto de reacción.

La influencia de la concentración de $CaCl_2$ en la fase acuosa de la mezcla de reacción fue otra variable estudiada. Para este propósito, se utilizaron diferentes concentraciones de $CaCl_2$ (17, 34 y 67 mmol/L), se observaron mejoras en la conversión con diferentes concentraciones de sustratos, y se determinó que la utilización de 17 mmol/l de $CaCl_2$ fue suficiente para lograr incluso una conversión de PC de 99% a bajas concentraciones de sustrato. Sin embargo, la concentración de $CaCl_2$ es más relevante en las reacciones con alta concentración de sustratos. La reacción con 67 mmol/l de $CaCl_2$, 83 mmol/L de PC y una relación molar PC: tirosol de 1:5, produjo mayor conversión de PC (97%) que las reacciones con menos $CaCl_2$, conversiones de 90% y 86% fueron obtenidos utilizando 34 y 17 mmol/L de $CaCl_2$ respectivamente. Por otro lado, utilizando la concentración más alta de $CaCl_2$ (67 mmol/L) y de PC (167 mmol/L) con una relación molar PC: tirosol de 1:5, también se consiguió una alta concentración de PT en el producto de reacción (157 mmol/l) con 94% de conversión. Aunque se ha descrito que el calcio es un requisito aparentemente no significativo para PLDs procedentes de microorganismos *Streptomyces* [64], han sido descritas varias reacciones de transfosfatidilación conteniendo de 40 a 100 mM de $CaCl_2$ disuelto en la fase acuosa [151, 181].

Una vez encontradas las condiciones más adecuadas para llevar a cabo la transfosfatidilación, dos concentraciones de PC 83 y 166 mmol / L fueron elegidas para su posterior escalado. La concentración de tirosol remanente no se consideró como un factor significativamente negativo, ya que, el tirosol fue recuperado del medio de reacción a partir de la fase de butirato de etilo mediante destilación a vacio.

Seguidamente se estudió la reutilización de la enzima, la reutilización es un factor clave con el fin de aumentar la productividad. El propósito de este estudio fue el de recuperar tanto la fase acuosa que contiene la enzima, como la fase de butirato de etilo que contiene tirosol sin reaccionar para ser reutilizados en un segundo ensayo

con una nueva carga de PC. Como paso previo al escalado en planta piloto, estas reacciones se llevaron a cabo aumentado 10 veces el volumen total de reacción.

Se estudió la recuperación enzimática a tres concentraciones diferentes de PC (167 mmol/L, 83 mmol/L y 21 mmol/L) y se valoraron tres variables; 1) la composición de las mezclas de reacción, 2) la separación de las diferentes fases por centrifugación, y 3) la preparación de la mezcla de reacción para el segundo ciclo de reacción.

Los medios de reacción fueron centrifugados cuando la reacción llego al equilibrio. Tras la centrifugación, se observó un sistema trifásico compuesto por el tampón, el butirato de etilo, y una fase sólida compuesta principalmente por el PT producido. La fase butirato de etilo, que contiene principalmente tirosol, se recuperó fácilmente en todos los casos, un 90 % del tirosol pudo ser recuperado para todas las concentraciones. Sin embargo, sólo el 55%, 60% y 80% de las fases acuosas fueron recuperados tras la centrifugación quedando mezclado con el PT (en las reacciones con 167 mmol/L, 83 mmol/L y 21 mmol/L de PC). Cabe señalar que cuanto mayor es la concentración de sustratos en la mezcla de reacción, más baja es la recuperación de ambas fases. Los segundos ciclos de reacción se prepararon en las mismas condiciones, pero estas reacciones alcanzaron conversiones significativamente más bajas que los primeros ensayos. Tan sólo se lograron conversiones de 13 %, 44 % y 80 % con 167 mmol/L, 83 mmol/L y 21mmol/L de PC al utilizar el medio enzimático reutilizado. Por lo tanto, sólo las reacciones con baja concentración de sustrato parecen ser las apropiadas para la reutilización de la enzima. Como se ha indicado anteriormente, la concentración de colina en la fase acuosa ha sido descrita como un inhibidor de la reacción[130], de este modo la reutilización de la enzima en transfosfatidilación requiere una etapa de eliminación de colina. Por otra parte, la reutilización de la enzima también se estudió en presencia de diferentes concentraciones de $CaCl_2$. El resultado obtenido fue diferente ya que, la fase acuosa se separó fácilmente después de la centrifugación (por encima de 90 % de la fase acuosa inicial). Sin embargo, como ya se ha descrito, estas condiciones de reacción con $CaCl_2$ producen una conversión más alta de PC y, en consecuencia, supondrá una mayor concentración de colina que la lograda en ausencia de $CaCl_2$. Por lo tanto, en presencia de $CaCl_2$, la producción de PT en los ciclos llevados a cabo con enzima reutilizada fue casi insignificante. Así que, la reutilización de la enzima quedó descartada, ya que para conseguir aislar la pequeña

cantidad de enzima del medio acuoso o para eliminar la colina de la fase acuosa serían necesarios otros métodos que comprometerían la simplicidad del proceso.

Seguidamente, se estudió la metodología para purificar el PT procedente de reacciones a tres concentraciones diferentes de PC (167 mmol/L, 83 mmol/L y 21 mmol/L). El método desarrollado fue la separación del fosfatidiltirosol formado mediante centrifugación a temperatura controlada. Después de la etapa de centrifugación, se observó, un sistema trifásico compuesto por una fase acuosa constituida principalmente por la disolución de tampón acetato utilizado, otra fase liquida de menor densidad formada principalmente por butirato de etilo y una interfase rica en el producto de la reacción (PT). Al igual que en la etapa previa de reutilización de la enzima, tras la centrifugación, la fase de butirato de etilo, contenía principalmente tirosol, que pudo ser recuperado con facilidad. Seguidamente se separó la fase acuosa del PT tras un segundo paso de centrifugación quedando parte del PT en la fase acuosa. Y después se efectuaron dos lavados de la interfase solida rica en PT: 1) lavado con agua para eliminar restos de sales y 2) lavado con butirato de etilo para eliminar restos de tirosol. Finalmente, el análisis por HPLC mostró que el aislamiento del PT a partir de la mezcla de reacción con la concentración más baja de PC (21 mmoles/L) dio el mejor resultado, dando lugar a un producto final con 84% en peso de PT y un rendimiento de extracción de PT del 54%. Sin embargo, a concentraciones más altas de PC (83 mmol/L y 167 mmol/L), se observó una difícil separación de la fase acuosa tras la centrifugación, consiguiendo productos con menos de un 75% de PT. Además, en estas condiciones el rendimiento de aislamiento PT fue también bajo. Cabe señalar que una pequeña cantidad de butirato de etilo quedo atrapado en la fase precipitada que produjo la contaminación parcial del PT con tirosol.

Para mejorar el aislamiento en medio de reacción con altas concentraciones de sustratos, se evaluó la influencia de $CaCl_2$ en el aislamiento del PT. Por lo tanto, se añadió $CaCl_2$ (67 mmol/l) a las reacciones para aumentar la fuerza iónica en la fase acuosa y cambiar la partición de PT entre ambas fases.

En estas condiciones de reacción con $CaCl_2$, se observó una gran mejora en la separación de fases, la fase acuosa se separó fácilmente tras la primera centrifugación (fue posible recuperar más del 90% de la fase acuosa inicial), y PT y la fase de butirato

de etilo fueron fácilmente separados en una segunda centrifugación. Como ya se ha señalado, con el $CaCl_2$ se produjeron conversiones de PC más altas, pero además, se mejoró el aislamiento de PT, lográndose un menor contenido de tirosol en el producto final que contenía 97 % de PT.

Sin embargo, de manera similar a lo observado en ausencia de $CaCl_2$, a la concentración de sustratos más alta estudiada (167 mmol/L de PC) y en presencia de $CaCl_2$, la separación de fases tras centrifugación, no llego a ser completa.

Tras la deshidratación del producto obtenido y para valorar la cantidad de sales en el producto final, se realizó una determinación del porcentaje de cenizas del producto final. Se comparó el contenido de sal entre la PC inicial y el PT producido mediante la determinación del porcentaje en peso de cenizas. Por otro lado, se determinó la humedad del producto final, siendo un 4 % el contenido en agua del PT purificado.

Con el fin de obtener mayores cantidades de PT altamente purificado, las dos etapas principales implicadas en el proceso (transfosfatidilación y purificación), fueron desarrolladas a escala planta piloto. En primer lugar, se llevó a cabo la reacción en un reactor de acero inoxidable de 1 litro de capacidad, con 660 g de mezcla de reacción. Seguidamente se utilizó una centrífuga en continuo termostatizada para obtener el producto. Tras la centrifugación en continuo, el PT quedó en el interior del cilindro de clarificación de la centrifuga. La Figura 12 muestra un diagrama de la producción de PT y el sistema de separación semi-continuo a escala planta piloto. Durante la centrifugación, el volumen del cilindro de clarificación (250 mL) se llena de fase acuosa y PT. Así se observó que volúmenes de fase acuosa y PT superior a 250 mL son arrastrados por la salida superior de la centrífuga de forma simultánea a la fase de butirato de etilo. Por esta razón, la escala elegida en planta piloto, fue la que da lugar a una mezcla de fase acuosa y PT de aproximadamente 250 mL. La reacción se compuso de más de 600 g de medio de reacción, con 83,3 mmol/L de PC, relación molar 1:5 PC: tirosol, 67 mmol/L de CaCl2, 211 mL de fase acuosa, y 422 mL de butirato de etilo usando 40 mg (1%) de PLD (1.500 unidades/mg).

En las condiciones de reacción elegidas, la mezcla se mantuvo 15 h y seguidamente el medio de reacción fue introducido en la centrífuga con la ayuda de una corriente de presión positiva de nitrógeno. La mayor parte de la fase de butirato de etilo se eliminó

por la salida superior y la fase acuosa se recogió a través de la purga de descarga. Por otro lado, el PT se recogió en el interior del cilindro de clarificación y se apartó. Las dos fases líquidas se volvieron a mezclar y se introdujeron en la centrífuga para recuperar el PT residual, que volvió a acumularse como una fase sólida en el cilindro de clarificación. Las dos fases sólidas se mezclaron, se secaron y molieron. Finalmente, tras su deshidratación, se produjeron 41 g de un polvo con 94 % de PT y 6 % de tirosol. El rendimiento del procedimiento de aislamiento de PT a esta escala fue mejorado, ya que fue del 86 %, y la humedad del 4 %.

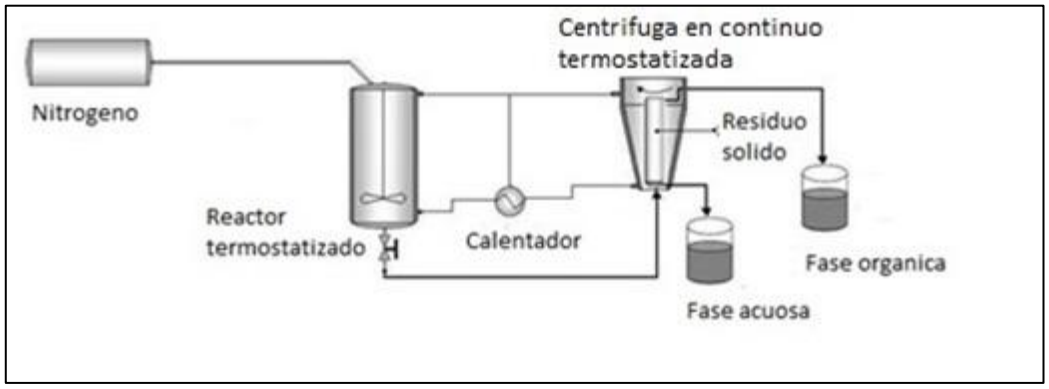

Figura 12. Diagrama proceso escala planta piloto

El principal problema encontrado en este novedoso proceso de producción de PT, fue el excesivo uso de tirosol, ya que, este sustrato queda sin reaccionar en el medio de reacción. La solución propuesta en el trabajo, consistió en la recuperación del tirosol procedente de la fase de butirato de etilo centrifugada mediante evaporación a vacío. No obstante, el exceso de uno de los sustratos resta viabilidad y rentabilidad a una posible aplicación industrial del proceso. Por lo tanto, en el artículo "Novel and efficient solid to solid transphosphatidylation of two phenylalkanols in a biphasic GRAS médium" se optimizó aún más, el proceso de producción. Se estudiaron los aspectos necesarios para lograr los objetivos propuestos en la producción del fosfolípido estructurado, tales como, eficiencia y productividad, además se incluyó también el estudio de producción del PHT.

El precio del HT disponible comercialmente es significativamente mayor que el precio del tirosol, esta es la principal razón por la que el trabajo se inició con tirosol y después se optimizó con los dos fenilalcanoles.

Los resultados obtenidos en el estudio de concentración de sustratos en las reacciones con tirosol y con HT son similares. La concentración de PC se incrementó desde 21 hasta 333 mmol/L con una relación molar 1:10 con ambos fenialcanoles. Pero la concentración de 333 mmol/L de PC fue excesiva y no se pudo formar ni PT ni PHT, considerando 167 mmol/L la concentración máxima de sustratos.

También se pudo observar una conversión de PC similar con los dos fenilalcanoles, al estudiar la relación molar entre sustratos. Sin embargo, se observó que la conversión de PC era algo peor para ambos fenilalcanoles empleando relaciones molares de PC: fenilalcanol de 1:3 y 1:2, lo que podría indicar, que la PC es el sustrato limitante en la reacción.

Seguidamente, se estudiaron altas concentraciones de sustratos con relación molar 1:2, con el fin de reducir al máximo posible el exceso de uno de los sustratos y abaratar así el proceso. Los resultados fueron positivos, pero en todas estas reacciones siguen permaneciendo cantidades importantes del fenilalcanol en exceso al final del bioproceso, lo que supone un alto coste sobretodo en HT. Además, complica el procesamiento posterior para la recuperación del nuevo fosfolípido producido. Por esta razón, la transfosfatidilación se estudió también con una relación equimolar de ambos sustratos a dos concentraciones diferentes, 83 y 167 mmol/L. Las conversiones de PC obtenidas en estas condiciones estequiométricas fueron significativamente bajas (60% y 35%) y la carga enzimática en estas reacciones se mantuvo al 1%. Otros autores, han descrito la relación entre la relación molar de sustratos al inicio de la reacción y el pH de la fase líquida [182], demostrando que incluso una relación entre sustratos ligeramente desigual puede tener un gran efecto en la tasa de reacción. Sobre la base de estos resultados, es posible crear un modelo para la predicción del cambio de pH desde el inicio de la reacción y durante el progreso de la misma, esto puede explicar tanto los rendimientos finales, como la cinética enzimática en estos sistemas [183]. Por lo tanto, el pH de estos sistemas puede ser controlado por la adición de sales inorgánicas (ácidos o bases). Estas sales pueden utilizarse también cuando la termodinámica en estas reacciones es desfavorable y no se logra la precipitación del producto. Además, la adición de contra-iones específicos que forman sales poco solubles con el producto (pero no con los sustratos) da lugar a sales de productos que precipitan en la mezcla de reacción. Tomando en consideración estos

hechos, se añadió 67 mmol/l de $CaCl_2$ a las mezclas de reacción con relaciones equimolares entre sustratos, lo que aumentó la conversión de PC hasta aproximadamente 85% en ambos fenilalcanoles, logrando así una mejora muy significativa.

A continuación, se investigó la proporción volumétrica entre la fase acuosa y la fase butirato de etilo, planteándola como una reacción sólido-sólido. Se ha señalado que la viabilidad termodinámica de las reacciones de sólido-sólido es independiente del solvente a una actividad de agua fija [184]. Cabe también señalar que en estas reacciones los sustratos no están completamente disueltos en el medio y los productos precipitan según se van formando, de este modo el producto se acumula en dos fases; el precipitado sólido y la fase líquida de la mezcla de reacción. Como regla general, se ha sugerido que los disolventes más bien hidrófilos (con bajo log P) dan los mejores resultados en reacciones sólido-sólido de péptidos y ésteres de AGs con azúcares [185]. Estas observaciones se explican por una correlación inversa entre el rendimiento del producto y la solubilidad del producto [186], por tanto, se estudió la influencia de la proporción volumétrica relativa al medio bifásico (relaciones volumétricas de butirato de etilo: tampón 10:1, 5:1, 2:1, 1:1, 1:2, 1:5, 1:10). Para conocer la relación optima del medio bifásico, la concentración de ambos sustratos elegidos fue 167 mmol/L. Los resultados indicaron que las proporciones de butirato de etilo: tampón óptimas, son diferentes según el fenilalcanol utilizado, para el tirosol fue (2:1) y para el HT (1:1). Por otra parte, en todos los casos estudiados, la conversión de la reacción fue menor para HT que la de tirosol. Estas diferencias observadas podrían estar relacionadas con la diferente solubilidad de tirosol e HT en butirato de etilo y tampón. El tirosol es menos polar (Log P 1.05 ± 0.34) [187], se solubiliza en butirato de etilo y además, no se ve afectado por la proporción de tampón en la mezcla de reacción cuando la proporción de butirato de etilo: buffer es menor que 2. Por el contrario, el HT más polar (Log P 0.55 ± 0.65) se puede repartir tanto en butirato de etilo como en tampón. Por esta razón, el aumento de las proporciones de butirato de etilo superior a la relación 1:1 diluye la concentración efectiva de HT y se observa una reacción de transfosfatidilación con menor conversión. Por el contrario, proporciones más altas de tampón en el caso de tirosol tuvieron un efecto insignificante en la reacción de transfosfatidilación.

Por otro lado, se determinaron las solubilidades de PC, PT, y PHT en butirato de etilo, la baja solubilidad de estos tres fosfolípidos en butirato de etilo indica que la transfosfatidilación se produce en un sistema de reacción sólido-sólido. Por tanto, se puede señalar que la tasa de solubilización de la PC en la mezcla de reacción, es el paso limitante en la reacción de transfosfatidilación. Además, el producto de reacción se acumula como un sólido insoluble en la mezcla de reacción, desplazando el equilibrio de la reacción hacia la formación del producto. Este hecho mejora la conversión de PC y también permite aumentar la productividad volumétrica del sistema de reacción mediante el uso de concentraciones altas de sustratos de hasta 167 mM.

La reacción no deseada de hidrólisis enzimática de PC asociado con la reacción de transfosfatidilación también fue considerada. Para ello se estudió la aparición de ácido fosfatídico en las mezclas de reacción. Este subproducto indeseable no se detectó mediante HPLC, en ninguna de las reacciones de transfosfatidilación investigadas, lo que indica que no existió hidrolisis asociada a la reacción de transfosfatidilación.

Finalmente, se estudió el curso de la reacción con el tiempo de las dos reacciones de transfosfatidilación en condiciones óptimas. Estas reacciones se llevaron a cabo a 167 mmoles/L de ambos sustratos y una proporción butirato de etilo: tampón de 2:1 y 1:1 para el tirosol y el HT respectivamente. Se observaron diferentes velocidades de reacción en las dos transfosforilaciones, la reacción con HT, mostró una velocidad de reacción más baja que la de tirosol. Esta variación, se puede explicar por la diferencia estructural en el anillo de benceno entre los dos fenilalcanoles. Se ha descrito anteriormente que el grupo meta-hidroxilo en el anillo de benceno podría inhibir ligeramente la transfosfatidilación de la PLD [149]. En ambos casos se alcanzó el equilibrio de reacción en aproximadamente 15 h con más de un 90% de conversión de PC, por lo tanto, altas concentraciones de PHT y PT (137 y 145 mmol/L) se consiguieron en relativamente poco tiempo, sin utilizar exceso de sustratos y cantidades de enzima adecuadas para aplicaciones industriales (relación de reactivos/enzima de 100/1).

El aislamiento del PT y del PHT se estudió en las reacciones consideradas como optimas, con 167 mmol / L de PC y del fenilalcanol, en un medio bifásico compuesto de butirato de etilo y tampón 2:1, y 1:1 para PT y PHT, respectivamente. Como paso previo al escalado en planta piloto estas reacciones se llevaron a cabo aumentado 10

veces el volumen de reacción. Para recuperar el PT y el PHT se utilizó el método de centrifugación descrito en el artículo previamente publicado. Se observaron resultados similares tras la centrifugación de las mezclas de reacción, un sistema trifásico compuesto por una fase acuosa, butirato de etilo y una fase sólida formada principalmente por PT o PHT. Tras separar las fases, la fase sólida obtenida, se lavó con butirato de etilo y agua destilada. Se obtuvieron dos productos sólidos con una composición de 94% de PT (6% tirosol y 0% de PC) y 91% de PHT (6% HT Y 3% de PC). El contenido en cenizas de los dos productos fue de 10%, y el contenido de agua 4% y 5% en PT y PHT respectivamente.

Finalmente, el proceso fue escalado de manera similar al PT obtenido antes de optimizar el proceso. Aunque en este caso, 204 mL de mezcla de reacción se incorporaron en el reactor de 1 L para probar la reproducibilidad del procedimiento a esta escala. La mezcla de reacción se centrifugó dos veces para eliminar la fases acuosa y el butirato de etilo, después, se lavó el sólido resultante con butirato de etilo y agua destilada para obtener PT y PHT con el menor contenido posible de tirosol e HT.

Finalmente, se obtuvieron 26 y 24 g de PT y PHT en polvo. Los productos estaban compuestos respectivamente de 88% de PT, 7% de tirosol, y 5% de PC en un caso y de 86% de PHT, 8% HT y 6% de PC en el otro. El contenido de cenizas fue 10% en ambos casos, y la humedad fue de 5% y 3% para PT y PHT respectivamente.

El rendimiento de los productos, definido como el porcentaje de peso de producto puro obtenido fue similar antes y después del escalado para PT y PHT (85% y 76%). Estos resultados indican que el procedimiento descrito es fácilmente escalable, aunque podrían investigarse más a fondo las etapas de lavado y centrifugación. Una pureza ligeramente inferior de PT y PHT se obtuvo al escalar a 204 mL en comparación a la de 24 mL. Este resultado podría estar relacionado con la geometría de los diferentes reactores y dispositivos de centrifugado utilizados que pueden influir tanto en la conversión como en la eficiencia de los lavados posteriores. Por el contrario, se obtuvo un contenido similar de fenialcanoles sin reaccionar en ambos procedimientos. Estos resultados sugieren que tanto la conversión de la reacción, como los procedimientos de lavado, deben ser considerados cuidadosamente durante el escalado con el fin de mejorar la pureza y eliminar la PC sin reaccionar de manera más eficiente. Sin

embargo, se debe tener en cuenta que un lavado más eficiente también podría conducir a rendimientos más bajos de PT y PHT por solubilización parcial de los productos de reacción durante los lavados. Dos técnicas de secado fueron empleadas: evaporación a vacío y liofilización. Ambas metodologías dieron lugar a productos con contenido en agua similar, lo que indica que las dos técnicas de deshidratación resultan adecuadas para eliminar la humedad de los productos finales.

CONCLUSIONES

8 CONCLUSIONES

Las conclusiones derivadas del trabajo realizado se clasifican según los objetivos planteados:

1. Se ha ilustrado la validez de los lípidos portadores estructurados en la mejora de bioactividad e incorporación de ingredientes funcionales en alimentos. Entre los diversos sistemas de administración de lípidos, los fosfolípidos han sido elegidos como lípidos portadores de interés. Las diferentes fosfolipasas y reacciones disponibles en el ámbito científico e industrial han sido revisadas. Esto ha permitido planificar un proceso de modificación de PLs viable para la industria alimentaria.
2. Se ha adaptado la metodología de extracción con disolventes y análisis de fosfolípidos ya existente, logrando un método de extracción y dos nuevos métodos de HPLC adecuados para la monitorización de las reacciones orientadas a la obtención de fosfolípidos estructurados. Además, se han desarrollado otros dos métodos de HPLC semipreparativo acoplado a colector de fracciones, que lograron purificar los nuevos fosfolípidos estructurados, para su uso como patrón analítico.

3. Se han obtenido dos fosfolípidos estructurados, de alta pureza que incorporan en su estructura un compuesto fenólico bioactivo. La producción a escala planta piloto de ambos productos ha sido llevada a cabo para su incorporación en alimentos como ingrediente funcional o tecnológico.

4. Se ha desarrollado un proceso biocatalítico (habitualmente caracterizado por el uso de disolventes) sin utilizar ningún componente potencialmente peligroso para el ser humano, integrando tecnologías inocuas y medioambientalmente limpias. En este proceso, se ha desarrollado un nuevo medio de reacción nunca antes descrito, compuesto de una fase acuosa y butirato de etilo, que puede

ser considerado como una alternativa a los disolventes orgánicos más tóxicos comúnmente utilizados.

5. Se ha conseguido desarrollar una eficiente reacción sólido-sólido de transfosfatidilación, optimizando parámetros de reacción como relación entre sustrato, concentraciones de sustrato, carga enzimática, composición del medio bifásico y contenido en sales, para lograr un proceso con alta productividad y un producto de elevada pureza.

6. Se ha asegurado la potencial viabilidad del proceso en el sector industrial alimentario basando su rentabilidad en una baja carga enzimática mediante el empleo de una relación enzima:sustratos de 1:100. El proceso además, se ha desarrollado utilizando una fosfolipasa D de grado alimentario.

7. Se ha diseñado un proceso de purificación del fosfolípido, producido de forma sencilla y sin utilizar disolventes tóxicos. Teniendo en cuenta que la reacción enzimática se llevó a cabo en condiciones equimolares y que el medio bifásico fue elegido teniendo en cuenta la purificación posterior del producto. Dicha purificación requiere tan solo la centrifugación del medio de reacción para recuperar el nuevo fosfolípido con elevada pureza y rendimiento.

8. Se ha ajustado el proceso desarrollado en planta piloto, valorando los problemas que surgen en el escalado. Además, este procedimiento es adecuado para preparar cantidades suficientes de estos dos nuevos fosfolípidos que permitan evaluar su potencial como antioxidantes tecnológicos, o investigar sus posibles actividades biológicas.

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